ANNUAL CYCLE IN GLYCEROL PRODUCTION AND CLEARANCE IN THE RAINBOW SMELT (Osmerus mordax) IS PARTIALLY REGULATED BY CYTOSOLIC AND MITOCHONDRIAL GLYCEROL 3-PHOSPHATE DEHYDROGENASE

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Annual Cycle in Glycerol Production and Clearance in the Rainbow Smelt (*Osmerus mordax*) is Partially Regulated by Cytosolic and Mitochondrial Glycerol 3-phosphate Dehydrogenase

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A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements

for the degree of Master of Science

Biochemistry Department and

Ocean Sciences Centre

Memorial University of Newfoundland

January 2010

St. John's Newfoundland, Canada

Abstract

In winter, rainbow smelt (*Osmerus mordax*) increase plasma glycerol concentrations to ≥400 mM to prevent freezing. Glycerol levels then decrease in the late winter/early spring. To enhance understanding of the biochemical mechanisms controlling changes in glycerol levels in rainbow smelt I: 1) examined the tissue distribution of cytosolic glycerol 3-phosphate dehydrogenase (cytGPDH) in the rainbow smelt, and two species that do not accumulate glycerol in the plasma for colligative freeze avoidance (the Atlantic salmon and capelin) when held at warm (10°C) and cold (~1 °C) temperatures; 2) established which cytGPDH isoforms are present in rainbow smelt liver and white muscle; and 3) examined the potential role of mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) in the late winter/early spring decrease in plasma glycerol levels. The tissue distribution study of cytGPDH confirmed the liver as the primary source of glycerol in the rainbow smelt, and cytGPDH does not respond to cold exposure in the Atlantic salmon and capelin. However, it also raised the possibility that muscle cytGPDH may contribute to glycerol accumulation in the smelt. Zymograms revealed differential cytGPDH regulation, with 4 isozymes in the liver and only 2 in the muscle of rainbow smelt. However, this distribution pattern was not affected by temperature (ie. glycerol production status). Higher activity levels of hepatic mGPDH were measured just before plasma glycerol levels returned to basal levels, and mGPDH mRNA expression was generally higher during the glycerol decrease than during the accumulation phase. Collectively, the results suggest that both cytosolic and mitochondrial GPDH enzymes play important roles in glycerol regulation.

Acknowledgments

I wish to thank my supervisor, Dr. W.R. Driedzic, for his constant encouragement, research input and open mind. The degree of professionalism and competence he embodies is a model for all his students. I am greatly indebted to a wonderful technical staff, Connie Short, Jennifer Hall, and Kathy Clow whom have been available at every facet of my research at the Ocean Sciences Centre. Their technical expertise is truly *second to none* and I greatly appreciate all their assistance. For animal collection I thank the Field Services Unit of the OSC. I would like to thank my past and present lab mates Delphine Ditlecadet, Genvieve Bilodeau, Jason Treberg, Simon Lamarre, and Erin Raynard for their support and assistance with equipment and assays. Furthermore, I thank the members of my committee, Dr. J.T. Brosnan and Dr. A.K. Gamperl for their experimental input as well as assistance with my scientific writing. I am grateful for my friends here at the OSC for making this such a welcoming place to do research as well as to Reanne Meuse for our 'friendship nights' and listening to me ramble on about science until we had it all figured out. Finally, I thank my parents and brothers for their encouragement and understanding as I continue to toil away at my curiosities.

My work has been supported financially by a grant from CIHR (WRD) as well as by a graduate fellowship from the School of Graduate Studies.

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Abbreviations

AFP –	antifreeze protein
AlaAT –	alanine aminotransferase
AspAT –	aspartate aminotransferase
cytGPDH –	cytosolic/NAD-linked glycerol 3-phosphate dehydrogenase
DG –	desalting gel
DHAP –	dihydroxyacetone phosphate
dsDNA –	double stranded DNA
DTT –	dithiothreitol
FFA –	free fatty acids
GA3P –	glyceraldehyde 3-phosphate
GA3Pase –	glyceraldehyde 3-phosphatase
GDP –	guanosine diphosphate
GK –	glycerol kinase
GPDH –	glycerol 3-phosphate dehydrogenase
GTP –	guanosine triphosphate
G3P –	glycerol 3-phosphate
INT –	iodoformazan
mGPDH –	mitochondrial/FAD-linked glycerol 3-phosphate dehydrogenase
PCR –	polymerase chain reaction
PEP –	phosphoenolpyruvate
PEPCK –	phosphoenolpyruvate carboxykinase

PK – pyruvate kinase

PMSF – phenylmethanesulphonylfluoride

TAG – triacylglycerol

TPI – triose phosphate isomerase

T3 – triiodothyroid hormone

qPCR – Quantitative PCR

WAT – white adipose tissue

1. Introduction

Glycerol (1,2,3-propanetriol) is a trihydric alcohol that is highly soluble in water. Glycerol is naturally found in organisms on its own or as the scaffold molecule for triacylglycerol (TAG). The level of glycerol is regulated in a variety of organisms and under numerous conditions, including stress responses such as hypo/hyperglycemia, low temperature, desiccation and anoxia. This introductory chapter details current knowledge regarding glycerol as an antifreeze molecule in rainbow smelt (*Osmerus mordax,* Mitchill, 1814), the regulation of glycerol levels, and physical properties of the glycerol molecule (Figure 1.1).

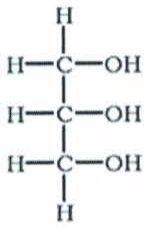

Figure 1.1: Structure of glycerol (1,2,3-propanetriol).

1.2. Glycerol accumulation and freeze avoidance in rainbow smelt

1.2.1. Seasonal glycerol/osmolyte production for use as a colligative antifreeze

Rainbow smelt produce massive amounts of glycerol in response to low temperature with plasma glycerol levels reaching 200-400 mM in the winter (Raymond, 1992). Glycerol levels begin to increase in late November, peak in March and decline to a typical teleost level (~1-5 mM) by mid-May (Figure 1.2) (Lewis et al. 2004). Through glycerol production, the rainbow smelt serum freezing point can reach as low as -2.0 °C in some instances (Raymond, 1992), which is lower than the freezing point of sea water (-1.8 °C). This is a great advantage for the rainbow smelt, as it not only allows them to live actively at subzero temperatures but also to

to feed on a myriad of invertebrates which, by nature of their physiology, have a lower freezing point than vertebrates. This enables rainbow smelt to access a protein source with almost no competition from other fish species.

While glycerol is the main freeze-avoidance osmolyte in rainbow smelt, antifreeze protein (AFP) (Ewart and Fletcher, 1990), trimethylamine oxide (TMAO), urea, and inorganic ions (Raymond, 1994) also increase in response to cold stress in this species.

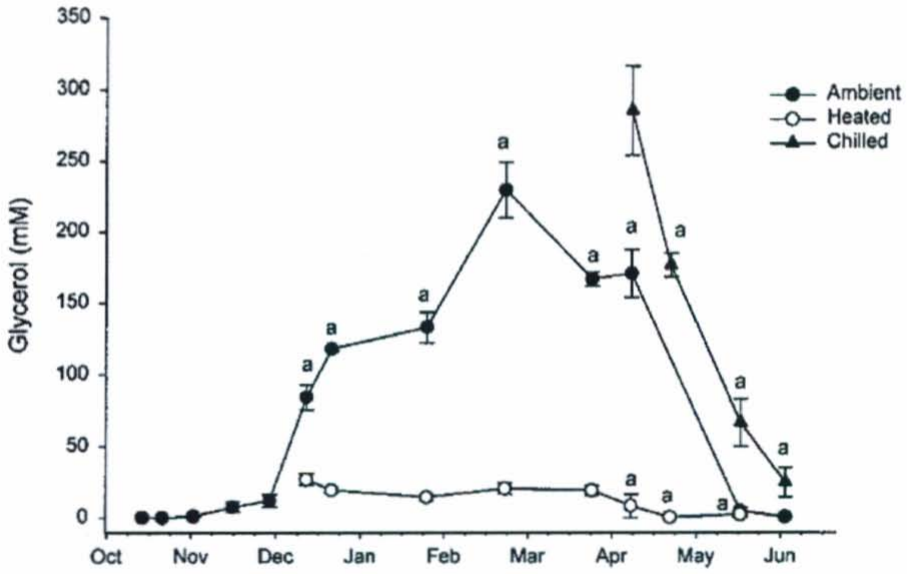

Figure 1.2: A comparison of seasonal plasma glycerol concentration in glycerol and non-glycerol producing rainbow smelt. Ambient refers to rainbow smelt maintained in sea water that followed natural temperature change. Heated refers to rainbow smelt maintained in 10 °C sea water. Chilled refers to fish artificially exposed to below normal temperature. "a" indicates significance (ANOVA: $p<0.05$) from initial sampling point within a treatment. Figure from Lewis et al. (2004).

1.2.2. Osmolarity effects of glycerol, TMAO, urea and inorganic ions

Maintenance of an appropriate osmotic pressure is crucial for the proper functioning of all cells. Most bony vertebrates regulate osmolarity using sodium, potassium, calcium, and chloride ions but, in some cases, osmolarity is also controlled by small organic solutes such as glycerol, urea, and TMAO (Treberg, 2002).

Rainbow smelt achieve their low-temperature-tolerance through elevations of plasma osmolarity to greater than 800 mOsmol kg^{-1}, an increase of more than 100% compared to summer levels (~350 mOsmol kg^{-1}) (Treberg et al. 2002a). The large increase in plasma osmolarity is associated with changes in glycerol, urea, TMAO, and minimal changes in inorganic ions (Raymond, 1992). Plasma urea and TMAO levels increased by approximately 19 and 9 mmol•L^{-1}, respectively, while glycerol was more than 150 mmol•L^{-1} greater in cold- versus warm-acclimated rainbow smelt (Treberg et al. 2002a). TMAO is synthesized from choline and betaine, and its production is hypothesized to take place in the kidney and potentially the liver (Treberg, 2002). Urea synthesis is common in fish, and elevated levels in rainbow smelt are likely the result of increased ammonia production from amino acid metabolism (Treberg et al. 2002a). Regardless, glycerol is considered the primary colligative antifreeze in rainbow smelt.

1.2.3. Antifreeze proteins (AFPs) act non-colligatively to inhibit ice crystal formation

AFPs work non-colligatively and irreversibly bind to ice crystals (Knight and DeVries, 1994). AFP binding arrests ice crystal growth thus increasing cold tolerance. AFPs have been described in rainbow smelt as well as many other teleosts inhabiting polar and subpolar oceans. The action of AFP is measured by thermal hysteresis, which is calculated from the freezing temperature minus the melting temperature. Thermal hysteresis in rainbow smelt via AFP provides only 0.3 °C of protection, insufficient on its own for life in -1.8 °C sea water since fish plasma typically freezes at ~ -0.7 °C (Ewart and Fletcher, 1990). Since AFP thermal hysteresis is many hundred-fold more effective than colligative osmolytes on a per molecule basis, AFPs actual role in rainbow smelt could turn out to be large during the glycerol decrease phase. However, AFPs are not the focus of this study and will only be referred to as is appropriate.

1.2.4. Glycerol in rainbow smelt comes from a variety of sources

Rainbow smelt require large amounts of carbon to drive the synthesis of plasma glycerol to levels >400 mM (Raymond, 1992) and do so by a variety of means. Fasted rainbow smelt can produce glycerol for short periods of time. Therefore, rainbow smelt can synthesize their own glycerol rather than relying on exogenous substrate. High glycogen phosphorylase activity and declining glycogen levels implicate glycogen as an immediate glycerol source (Clow et al. 2008). However,

stored levels of glycogen are insufficient to provide the glycerol levels observed in cold-acclimated individuals (Raymond, 1995; Raymond et al. 1996; Treberg et al. 2002b). Therefore, rainbow smelt must obtain carbon from a different source to maintain glycerol levels throughout the winter.

Triglyceride pools are a potential source of glycerol because their three carbon backbone could be released to provide glycerol. Raymond et al. (1996) investigated this potential glycerol production pathway and found triglyceride concentrations *increased* in cold-acclimated rainbow smelt relative to warm-acclimated rainbow smelt which do not accumulate glycerol. Therefore, carbon sources other than triglyceride are used for glycerol synthesis.

Radiolabelling studies have demonstrated that amino acids are the preferred carbon source for glycerol production in rainbow smelt, even when presented with glucose (Walter et al. 2006). The utilization of amino acids is an obvious solution as rainbow smelt tolerate icy cold temperatures to capitalize on a vast protein food source. The available protein is degraded into its component amino acids and used to synthesize glycerol through a pathway called glyceroneogenesis (Section 1.3). For example, alanine and aspartate can be enzymatically converted, into pyruvate and oxaloacetate, respectively. Since many amino acids are gluconeogenic it follows that they are also glyceroneogenic.

1.2.5. Activation of glycerol production

The production of glycerol is a tightly regulated process. Cold water temperatures trigger high glycerol production in the rainbow smelt and as temperatures warm in the spring, glycerol production and plasma glycerol concentrations drop to a typical teleost level (Lewis et al. 2004). This *in vivo* phenomenon is supported by analysis of glyceroneogenic enzymes (Section 1.3). In order for glycerol to be produced from amino acids carbon must flux through phosphoenolpyruvate carboxykinase (PEPCK) and cytosolic glycerol 3-phosphate dehydrogenase (cytGPDH) (Figure 1.3). Expression analysis (using qPCR) of these two protein transcripts during glycerol production, independently showed spikes in early to mid December (between ~5 and 2 °C) with glycerol concentrations substantially increasing during this time. PEPCK sustains a high level of expression for approximately one month, after which its expression decreases rapidly. The decline in PEPCK expression is followed by a delayed drop in plasma glycerol level. cytGPDH expression levels quickly drop after the December spike while plasma glycerol levels continue increasing into March (Liebscher et al. 2006). Furthermore, the hepatic activity of cytGPDH in late winter has been shown to be significantly higher in cold-acclimated relative to warm-acclimated rainbow smelt. This suggests that high quantities of cytGPDH are synthesized and maintained for glycerol production (Lewis et al. 2004).

Clow et al. (2008) isolated hepatocytes from warm-acclimated rainbow smelt (8°C) and found that exposure of cells to cold temperature (0.4°C) alone

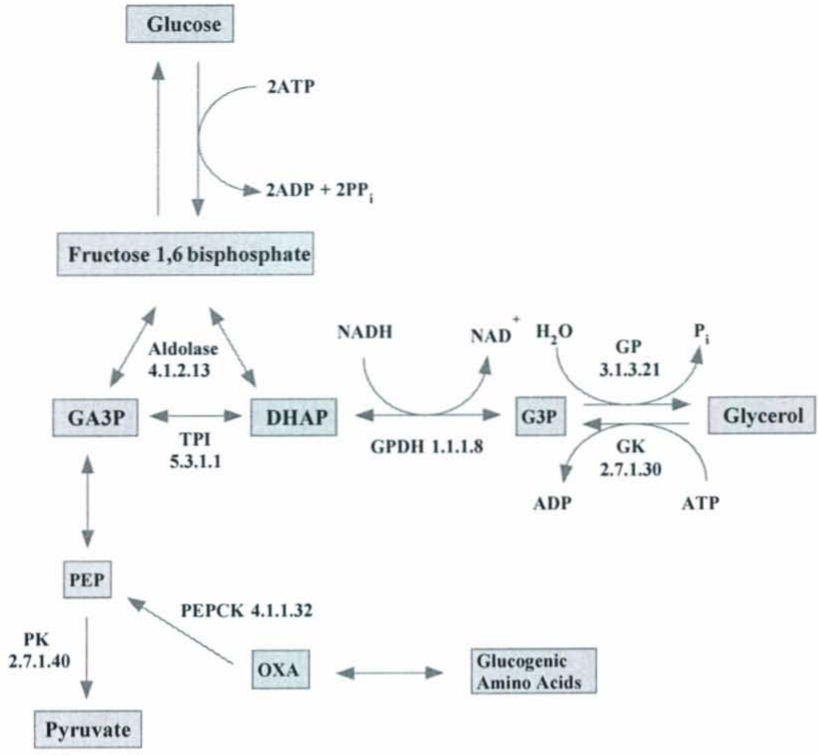

Figure 1.3: A schematic diagram detailing key enzymes and metabolites involved in glycerol synthesis and catabolism. Intermediates are in boxes and are abbreviated as follows: PEP (phosphoenolpyruvate), GA3P (glyceraldehyde 3-phosphate), DHAP (dihydroxyacetone phosphate), G3P (glycerol 3-phosphate). Enzymes catalyzing specific reactions are abbreviated followed by their respective enzyme classification number. TPI (triose phosphate isomerase), GPDH (glycerol 3-phosphate dehydrogenase), GK (glycerol kinase), PEPCK (phosphoenolpyruvate carboxykinase), PK (pyruvate kinase), GP (glycerol phosphatase).

caused a shift from the production of glucose to high levels of glycerol. However, attempts to terminate *in vitro* glycerol production in hepatocytes have not been successful and it is likely that factors other than temperature play a role in the down regulation of glycerol production, but this has yet to be shown (Clow et al. 2008).

1.2.6. Glycerol loss and replenishment

Glycerol production is an energetically expensive process, as it requires valuable carbon precursors such as glucose, pyruvate and amino acids. Given that these molecules are central to cell metabolism it would seem like an energetic waste to release endogenous glycerol to the external environment. However, glycerol is continually lost (up to 10% per day; ~2.1 J/100 g^{-1} day^{-1}) through the skin, gut, and gills of the rainbow smelt (Raymond, 1993; Raymond, 1995). Glycerol must be constantly replenished; therefore, glycerol production is a continuous process, requiring a constant supply of substrates and cofactors. As a consequence, rainbow smelt need to eat about twice as much during thermal decline (Raymond, 1995). Radiolabelled studies have shown that more than half of injected alanine and glutamate were used for glycerol and glucose production. Cold-acclimated rainbow smelt given exogenous amino acids did not deplete glycogen reserves (Raymond and Driedzic, 1997), whereas fasted fish rapidly mobilize glycogen (Driedzic and Short, 2007). It appears that on a high protein diet, metabolizing amino acids to glycerol is the most efficient way to sustain this crucial osmolyte.

The process of seasonal glycerol decrease requires further investigation as there is currently no physiological explanation for the loss. Lewis et al. (2004) monitored plasma glycerol levels in rainbow smelt from October to June. In mid-February to May, the plasma glycerol level in rainbow smelt dropped by >200 mM. A decrease of this magnitude is greater than can be explained by the glycerol loss mechanisms mentioned above (Raymond, 1993).

1.3. Glyceroneogenesis

1.3.1. Glyceroneogenesis functions to synthesize glycerol

Glyceroneogenesis is a truncated gluconeogenic pathway and is responsible for the *de novo* synthesis of glycerol/glyceride from sources other than glucose (Figure 1.3). In order for glyceroneogenesis to function properly, key enzymes must be present to convert amino acids and Krebs cycle intermediates to glycolytic substrates. Cold-acclimated rainbow smelt undergo substantial glyceroneogenesis through mobilization of amino acids to glycerol in liver (Raymond et al. 1997).

1.3.2. Phosphoeneolpyruvate carboxykinase is the key glyceroneogenic enzyme

Phosphoenolpyruvate carboxykinase (4.1.1.32) (PEPCK) functions to catalyze the GTP-driven decarboxylation of oxaloacetate to form phosphoenolpyruvate (PEP), while dephosphorylating GTP to GDP. This reaction bypasses the irreversible formation of pyruvate, that occurs via pyruvate kinase (PK), produced during glycolysis. Therefore, pyruvate must first be transformed to oxaloacetate (via pyruvate carboxylase) before it can be used to form glycerol/glucose. PEPCK is considered the rate limiting enzyme in glycero- and gluconeogenesis and, in mammals, is upregulated in both liver and adipose tissues during fasted/diabetic conditions when cellular glucose uptake is minimal (Reshef et al. 2003). PEPCK has been described in both the mitochondria and the cytosol but its sub-cellular location is unknown in rainbow smelt. *In vitro* activities of this enzyme have been shown to be higher in rainbow smelt than the closely related capelin, a non-glycerol accumulating species

(Treberg et al. 2002b). Rainbow smelt PEPCK expression has also been implicated in the onset of glycerol production, as described above (Liebscher et al. 2006).

1.3.3. Aminotransferases convert amino acids to Krebs' cycle intermediates

Rainbow smelt use amino acids as their carbon source for glyceroneogenesis (Raymond and Driedzic, 1997; Walter et al. 2006). Amino acids are metabolized in a variety of ways depending on the specific amino acid under consideration. Aspartate aminotransferase (AspAT) catalyzes the transamination of aspartate and the release of the α-ketoacid, oxaloacetate. Alanine aminotransferase (AlaAT), by a similar mechanism, converts alanine to pyruvate which can be converted to oxaloacetate for production of glycerol by way of PEPCK. In rainbow smelt, AlaAT and AspAT have higher activities than capelin (Treberg et al. 2002b). High AspAT and AlaAT activity could be due to increased malate-aspartate shuttle activity. However, low malate dehydrogenase and malic enzyme activities in rainbow smelt suggest that increased aminotransferase activities are due to glyceroneogenic flux (Treberg et al. 2002b). Furthermore, in radiolabelling studies on the rainbow smelt, ^{13}C labeled amino acids are converted to glycerol with adjacent amino acid carbons retained in the same position within the glycerol molecule. This further demonstrates that glycerol is derived directly from amino acids in the rainbow smelt (Walter et al. 2006). Despite the lack of evidence for all amino acids, it is probable that all glucogenic amino acids, through aminotransferase activity, contribute to glycerol maintenance in the rainbow smelt.

1.3.4. Glycerol 3-phosphate dehydrogenase activity in rainbow smelt is indicative of glyceroneogenesis

Cytosolic glycerol 3-phosphate dehydrogenase (1.1.1.8) (cytGPDH) is a bi-substrate enzyme catalyzing the reversible reduction of dihydroxyacetone phosphate (DHAP) yielding the sugar alcohol, glycerol 3-phosphate (G3P). In rainbow smelt, increased activity of cytGPDH is associated with glyceroneogenesis during cold temperature exposure. The glyceroneogenic path produces glyceraldehyde 3-phosphate (GA3P) which must first be transformed to DHAP via triose phosphate isomerase (TPI). Activity levels of TPI have not been measured in rainbow smelt, but it is assumed that they would be high since GA3P conversion to DHAP occurs exclusively through TPI (Richard, 2008).

Glycerol can also theoretically be synthesized from GA3P by way of glyceraldehyde 3-phosphatase (GA3Pase). Driedzic et al. (1998) found GA3Pase activity was not discernible in cold-acclimated rainbow smelt. However, cytGPDH activity levels in cold-acclimated individuals were 12– and 28–fold higher than flounder (*Liopsetta putmani*) and tomcod (*Microgadus tomcod*) respectively, two non glycerol accumulating teleosts from the same habitat (Driedzic et al. 1998). Treberg et al. (2002b) found hepatic cytGPDH activity to be significantly higher in glycerol-producing rainbow smelt than warm-acclimated individuals (Treberg et al. 2002b). Moreover, the maximal *in vitro* cytGPDH activity in cold-acclimated fish correlated with plasma glycerol concentrations during seasonal temperature drop. This suggests that cytGPDH activity in cold-acclimated fish is associated with the initial

glycerol increase (Lewis et al. 2004). Therefore, cytGPDH activity is essential, and indicative of major glycerol production in rainbow smelt.

cytGPDH has numerous functions and is found in all organisms (Bewley and Cook, 1990). cytGPDH transcripts are present in skin, gill, heart, head kidney, brain and liver in both rainbow smelt and Atlantic salmon as well as the spleen in rainbow smelt (Ewart et al. 2001). Despite the abundance of cytGPDH transcripts in rainbow smelt tissues, only liver has been implied as a site of glycerol synthesis based on enzyme activity levels. This is an issue that needs to be addressed to determine the whole body mechanism of glycerol production in rainbow smelt.

In most diploid teleosts cytGPDH is coded for by two separate gene loci A^* and B^*. These loci code for the two homodimers, A_2 and B_2 and the heterodimer AB. The two loci are differentially distributed with the A^* locus most active in skeletal muscle and the B^* locus most active in the liver (Phillip et al. 1979, Fisher et al. 1980, Basaglia and Cucchi, 1993). Furthermore, electrophoretic analysis has revealed the presence of two A_2 isozymes, A''_2 and A'_2, indicating the possible presence of two co-dominant alleles at the A^* locus (Basaglia and Cucchi, 1995).

The regulatory role of cytGPDH has yet to be entirely understood. Different cytGPDH isozyme patterns occur in response to: growth and development in fish (Phillipp et al. 1979); neoplasia in rabbit tissues (Ostro and Fondy, 1977); environmental toxins in teleosts (Basaglia and Cucchi, 1995); dietary carbohydrate levels (Kang et al. 1999), and tissue type and sex in fruit flies (Stroppa et al. 2008). However, there is no concise regulatory description for the multifunctional cytGPDH

isozyme. cytGPDH isozyme number and pattern of expression has yet to be assessed in rainbow smelt. Since cytGPDH is so important to the survival of rainbow smelt, elucidating the tissue distribution and isozyme pattern could prove important.

1.3.5. FAD-linked GPDH

FAD-linked GPDH (mGPDH, EC. 1.1.99.5) is located on the outer face of the inner mitochondrial membrane (Klingenberg, 1970) and is named solely for the fact that its substrate is G3P. However, mGPDH is a much different protein than cytGPDH. mGPDH is also referred to as flavoprotein dehydrogenase or ubiquinone oxidoreductase, because of its position in the glycerophosphate shuttle, which works to bring reducing equivalents into the mitochondrial inner membrane for electron transport. mGPDH is similar to succinate dehydrogenase (electron transfer chain complex II) in that it reduces FAD to $FADH_2$. Sequence data on the mGPDH protein in rats shows that the protein has three transmembrane helices. The first membrane-spanning region contains the FAD binding site and is located within the mitochondrial membrane. The second region is located in the cytosol and contains both a calcium and G3P-binding domain, suggesting that cytosolic calcium and G3P can regulate mGPDH action (MacDonald and Brown, 1996).

mGPDH is a well known protein with multiple regulatory functions at the junction of fat and carbohydrate metabolism. Currently no information exists detailing mGPDH's role in rainbow smelt during the phases of glycerol production and decrease.

1.4. Conclusion

Model organisms are particularly useful to enhance our understanding of metabolism. The magnitude and predicability of glycerol production in rainbow smelt make them a prime model species to exploit for the investigation of glycerol metabolism. This study investigates cytGPDH in rainbow smelt to determine if tissues other than the liver are producing glycerol, if multiple isozymes of the cytGPDH enzyme exist in muscle and liver, and the seasonal transcriptional regulation of this enzyme. Furthermore, this study examines seasonal mGPDH activity, regulation and expression for the first time in the rainbow smelt. The approach of comparative biochemistry is taken through the utilization of non-glycerol accumulating species, the Atlantic salmon and capelin, as compared to warm- and cold-acclimated rainbow smelt.

2. Glycerol 3-phosphate dehydrogenase (GPDH) activity levels, isozyme number, and GPDH expression in rainbow smelt liver and other selected species and tissues

2.1. Introduction

The rainbow smelt (*Osmerus mordax,* Mitchill, 1814) is an anadromous teleost species that accumulates plasma glycerol levels to >400 mM in response to cold temperatures to avoid freezing (Raymond, 1992). Other osmolytes are also produced by cold-acclimated rainbow smelt such as TMAO (Treberg et al. 2002a), urea, and inorganic ions (Raymond, 1992). However, glycerol is the predominant osmolyte.

Glycogen is important for generating the initial glycerol surge but rainbow smelt must actively feed to maintain high glycerol levels throughout cold temperature exposure (Driedzic and Short, 2007). Studies using injected radioisotopes reveal that glycerol is derived from exogenous amino acids and glucose (Raymond, 1995; Raymond and Driedzic, 1997; Walter et al. 2006). In order for glycerol to be synthesized, through glyceroneogenesis, it must flux through glycerol 3-phosphate dehydrogenase (cytGPDH) (EC: 1.1.1.8). cytGPDH is an NAD-linked bi-substrate enzyme that catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to glycerol 3-phosphate (G3P). Rainbow smelt prepare for glyceroneogenesis through a transient increase in cytGPDH mRNA expression in liver, as temperatures decline in December (Liebscher et al. 2006). Moreover, the activity of liver cytGPDH correlates with plasma glycerol concentration in rainbow smelt held in ambient sea water (Lewis et al. 2003, Driedzic et al. 2006, and Treberg et al. 2002b). However, a void exists in our knowledge as cytGPDH expression has not been determined in warm-acclimated, non-glycerol producing, rainbow smelt and to date all investigation into glycerol accumulation has been exclusive to liver or cultured hepatocytes. Therefore, it is not

known whether other tissues are playing an active role in glycerol accumulation and if cytGPDH expression is specifically regulated by temperature. Transcripts of cytGPDH are found in heart, kidney, brain, skin, spleen, and muscle in rainbow smelt. Therefore, all are possible sites of glycerol synthesis through activity of cytGPDH (Ewart et al. 2001).

cytGPDH, in all species evaluated to date, is found as a series of isozymes consisting of two subunits (see for example: Park et al. 2001, Stroppa et al. 1998, Fisher et al. 1980, Wilanowski et al. 1998). In teleosts, cytGPDH is coded for at two separate loci A^* and B^* that translate into dimerized proteins. Electrophoretic analysis has shown the presence of four possible teleost cytGPDH isozymes. These isozymes consist of two co-dominant alleles at the A^* locus (A''_2 and A'_2), a B_2 dimer formed from the B^* locus and a hybrid dimer of the A^* and B^* loci called AB (Basaglia and Cucchi, 1995, Phillipp et al. 1979, Fisher et al. 1980, Basaglia and Cucchi, 1993). The putative protein data is consistent with genetic data describing 4 translated *Drosophila melanogaster* cytGPDH transcripts (Wilanowski et al. 1998). Differential regulation of cytGPDH isozymes occurs in response to stress in numerous organisms (see for example: Phillipp et al. 1979; Ostro and Fondy, 1977; Basaglia and Cucchi, 1995; Kang et al. 1999; Stroppa et al. 2008), and therefore, is expected to be regulated in the glycerol-producing rainbow smelt.

This study examines 3 unknown aspects of teleost cytGPDH biochemistry. First, a tissue distribution of maximal *in vitro* cytGPDH activity was performed on cold- and warm-acclimated rainbow smelt, as well as, the non-glycerol accumulating Atlantic salmon (*Salmo salar*) (Fletcher et al. 1988) and capelin (*Mallotus villosus*) (Raymond et al. 2000). Second, the presence of cytGPDH isozymes in rainbow smelt white muscle and liver were

evaluated and compared. Finally, liver cytGPDH expression levels were measured for both warm- and cold-acclimated rainbow smelt throughout the winter season.

2.2. Materials and Methods

2.2.1. Animals and tissue sampling

Rainbow smelt used in the tissue distribution study for the analysis of cytGPDH enzyme activity were collected in mid-November 2006 from Mount Arlington Heights, Placentia Bay, Newfoundland. Fish were collected by seine netting in freshwater and brought to the Ocean Sciences Centre at Memorial University where they were moved to ~10 °C sea water. The rainbow smelt were randomly sorted into two separate tanks, an ambient temperature and a warm temperature tank, held on a natural photoperiod with fluorescent lights regulated by an outdoor photocell. Rainbow smelt held in the ambient tank followed natural temperature fluctuations with untreated sea water pumped from Logy Bay. Fish sampled from this group are considered to be cold-acclimated. The warm tank was also filled with running sea water from Logy Bay, but the water was heated to ~10 °C for the duration of the experiment. Fish sampled from this group are considered warm-acclimated. Fish from both groups were fed chopped herring 2-3 times per week and were randomly selected independent of sex or size on March 29 and April 13, 2007. Fish were bled through caudal puncture with heparanized syringes prior to killing by a sharp blow to the head. The heart, liver, muscle, kidney and brain were immediately harvested and flash frozen in liquid N_2. Whole blood was centrifuged for 5 min at 10,000 x g and the plasma was collected. Tissues and plasma were stored at -80 °C for future analysis.

Atlantic salmon (*Salmo salar*) were purchased from Cooke Aquaculture, Daniel's Harbour, N.L., maintained at the Ocean Sciences Centre on a natural photoperiod, and fed commercially available 3.5 mm pellets (EWOS, Surrey, BC). The Atlantic salmon were sorted into two tanks (warm and cold) on January 7, 2008 with the warm tank set at ~10 °C and the cold tank set at 5 °C. The temperature of the cold tank was dropped to 3 °C on January 16, 2008, and finally to 1 °C on January 18, 2008. On January 28 and February 29, 2008 salmon tissues and plasma were harvested and flash frozen after sacrifice (as above). Tissues were stored at -80 °C for future analysis. Capelin were collected from Middle Cove, Newfoundland with a seine net on July 5, 2007 and placed into a holding tank at ambient temperature (8 °C). The following day capelin were sacrificed and tissues and plasma were harvested, flash frozen, and stored at -80 °C.

For isozyme studies, rainbow smelt were collected in mid-November 2007 and held as above. Sampling took place on January 14, February 18, and March 27, 2008. Liver and muscle harvest was the same as in the enzyme distribution study conducted over the 2006 and 2007 seasons.

Rainbow smelt used in cytGPDH mRNA expression studies were collected in November 2008, held as described above and sampled approximately every 30 days until May 2009. Cold- and warm-acclimated rainbow smelt were sampled on the same day. Plasma samples were taken and the fish were killed by a sharp blow to the head. The liver was excised and ~100 mg was flash frozen in liquid N_2. The water temperatures of the two tanks were recorded for the season (Figure 2.1).

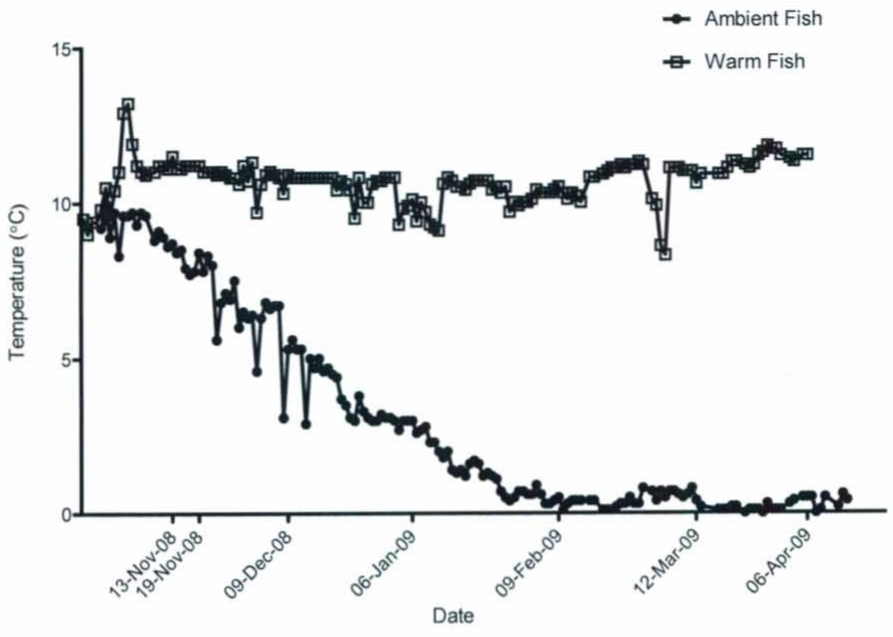

Figure 2.1: The seasonal water temperatures for cold- and warm-acclimated rainbow smelt tanks for the 2008-2009 winter season. Dates of fish sampling are shown on the x-axis.

2.2.2. Plasma glycerol concentration

Plasma glycerol concentration was determined using the Sigma diagnostic kit 33740A (Sigma-Aldrich, Oakville, ON) and plasma samples were diluted to remain in the linear range of the assay. The available glycerol is catalyzed by the assay medium to DHAP as well as peroxide. The formed peroxide, as well as 4-aminoantipyrine + N-ethyl-N-(3-sulfopropyl) m-anisidine (found in assay medium), are catalyzed by peroxidase to form a quinoneimine dye which absorbs maximally at 540 nm. Plasma plus reaction medium was incubated at room temperature for 15 minutes and the absorbance was read directly at 540

nm using a Beckman Coulter 880 Multimode Detector (Beckman-Coulter, Fullerton, CA). Samples were measured in duplicate and glycerol concentration was calculated using the provided standards.

2.2.3. NAD-linked GPDH activity assay

The cytGPDH activity assay was adapted from Joanisse and Storey (1994). Samples of liver, heart, brain, muscle, and kidney tissue were weighed and homogenized into 9 volumes of ice cold buffer (20 mM imidazole, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 0.1 mM PMSF, 5 mM DTT, pH 7.4). The buffer was designed to inhibit protein phosphatases (NaF), protein kinases (EGTA, EDTA), and proteases (PMSF) during homogenization and experimentation. The homogenate was centrifuged for 2 min at 2,000 x g to remove cellular debris. The supernatant was aspirated, transfered to a new tube, and maintained on ice. The assay consisted of tissue homogenate in the presence of 20 mM imidazole and 0.15 mM NADH at pH 7.2. The cytGPDH reaction was initiated by the addition of 1.87 mM DHAP. Absorbance was measured at 340 nm for ~10 min with a Beckman Coulter (DU 640) spectrophotometer to observe the conversion of NADH to NAD^+ by cytGPDH at 20 °C.

2.2.4. Identification of cytGPDH isozymes

Rainbow smelt liver and muscle were taken from the -80 °C freezer and homogenized in 9 volumes of extraction buffer (20 mM imidazole, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 0.1 mM PMSF, 5 mM DTT, pH 7.4). Homogenized samples were centrifuged for

2 min at 2,000 x g to remove cellular debris. The supernatant was transferred to a new tube and the proteins were salted in overnight with 50 mM ammonium sulphate at 4 °C. Salted in samples were centrifuged for 10 min, 10,000 x g at 4 °C. 3 mL of supernatant was aliquoted into a 10 DG desalting column (Bio-Gel P-6 Desalting Gel, Biorad, Hercules, CA) and the void volume was eluted as waste. The 10 DG column protein buffer was exchanged through the addition of 4 mL of 50 mM K_2PO_4, the eluent was collected and the protein concentration was determined using the Bradford method (Bradford, 1976).

Affi-gel Blue Gel (Biorad, Hercules, CA) columns were manually packed with ~5 mL of blue gel per column. ~20 mg of protein was added to the Affi-gel blue gel columns to interact with the cibacron blue molecule, that is attached to agarose beads in the Affi-gel blue gel slurry (Biorad, Hercules, CA). Samples were washed with two bed volumes of 50 mM K_2PO_4. Bound fractions were competitively eluted and collected using 5 mL of elution buffer (10 mM NADH, 1 M NH_4Cl, pH 7.4, 4 °C). Protein concentrations were again measured.

GPDH isozymes were visualized using cellulose-acetate gel electrophoresis following the staining procedure from Rothe (1994). Samples were added to Titan III cellulose acetate gels and placed in a Titan Gel horizontal chamber (Helena Laboratories, Beaumont, TX) for 45 min at 0.98 mA, 116 v. Gels were put into 50 mLs of incubation media (100 mg G3P, 200 mg pyruvic acid, 20 mg NAD^+, 1 mL MTT and PMS (5 mg/mL), 0.5% agarose, TRIS-HCl, 45 °C, pH 7.4) in a dark oven at 37 °C for 45 minutes. During the course of the reaction cytGPDH converts the available G3P to DHAP, which in turn generates NADH. The NADH is immediately and irreversibly oxidized by MTT and PMS to form a formazan

molecule. The formazan molecule is visible with the naked eye and represents the presence of the cytGPDH enzyme. The reaction was stopped by flooding the gel with 10% acetic acid. The presence of cytGPDH protein was represented by bands where reaction had taken place. Gels were briefly washed in water and imaged using a Syngene G:Box imager (Syngene, Frederick, MD).

2.2.5. RNA extraction

Total RNA was extracted from frozen liver using TRIzol reagent (Invitrogen, Burlington, ON) according to the manufacturer's instructions. Briefly, the tissue was homogenized with a Kontes RNAse-free pestle grinder (Kimble Chase, Vineland, NJ) for ~20 sec in 400 µL of TRIzol. A second aliquot of TRIzol (600 µL) was added to the tubes and mixed by inversion. The samples were placed at -80 °C for a maximum of 4 weeks before extraction.

Samples were removed from the freezer, thawed at room temperature, and centrifuged at 12,000 x g for 10 min at 4 °C. Chloroform was added (200 µL) and the tubes were shaken by hand for ~15 sec, and allowed to incubate for 3 min at room temperature. The tubes were centrifuged at 12,000 x g for 15 min at 4 °C, to separate organic and non-organic phases. The clear organic phase was extracted and added to new tubes containing 500 µL of isopropanol, and mixed through inversion. The tubes were again incubated at room temperature for 10 min, allowing the isopropanol to precipitate the RNA. Samples were centrifuged for 10 min at 12,000 x g and the supernatant was aspirated and discarded. 1 mL of 75% ethanol was added to the pellet and flicked to ensure that the pellet was im-

mersed in the ethanol. Samples were centrifuged for 5 min at 7,600 x g (4 °C), to wash the RNA. The supernatant was discarded and the pellet was re-centrifuged at 7,600 x g for 1 minute to remove any remaining ethanol. The RNA pellet was dried for 10 min and re-hydrated with 100 - 500 µL of ddH_2O (depending on pellet size). The RNA pellet was re-suspended by heating to 55 °C and pipetting up and down. RNA samples were stored at -80 °C for further use.

RNA concentration was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Furthermore, RNA preparations were quality controlled through the A_{260}/A_{280} ratio, and were loaded into 1% agarose gels for 30-45 min at 150 volts (with ethidium bromide).

2.2.6. DNAse treatment and cDNA synthesis

After thawing, the RNA samples were treated with DNAse to remove any contaminating genomic DNA. The DNase enzyme cleaves dsDNA non-specifically to leave 5' phosphorylated oligodeoxynucleotides. Removal of DNA was with the commercially available Turbo DNA-free (Ambion, Austin, TX) kit. Briefly, 10 µg of RNA was added to 5 µL of 10X TURBO DNase buffer and 1 µL of TURBO DNase (2 Units) and ddH_2O was added to achieve a final volume of 50 µL. Samples were incubated at 37 °C for 30 min and a second 1 µL aliquot of TURBO DNase was added (4 Units total). Samples were again incubated for 30 min at 37 °C. 10 µL of DNase inactivation reagent was added, tubes were well mixed, and incubated at room temperature for 2 min. Tubes were centrifuged at

10,000 x g for 1.5 min, and the resulting supernatant (purified RNA) was transfered to a new tube, and stored at -80 °C for future cDNA synthesis.

First strand cDNA was synthesized using M-MLV Reverse Transcriptase and random primers (Invitrogen, Burlington, ON). Briefly, 1 μg of DNAse treated RNA was added to 250 ng of random primers, 1 μL of 10 mM dNTP's, and enough ddH_2O to achieve a 12 μL volume. Tubes were heated at 65 °C for 5 min, briefly spun and put on ice. 4 μL of 5X First-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH 8.3) and 2 μL of DTT were added to each tube, followed by mixing and a 15 min incubation at 37 °C. Reverse transcriptase was added as 1 μL (200 U) of M-MLV RT, the tubes were mixed, and incubated at room temperature for 10 min. Tubes were incubated for 50 min at 37 °C to allow the reverse transcriptase enzyme to synthesize cDNA from the nascent mRNA strands. M-MLV RT is a non-specific enzyme that acts to enrich the cDNA concentration in the mRNA isolates for use in PCR. The reaction uses primers specific for the poly-adenosine tail, a characteristic of mRNA. The synthesis of cDNA is a crucial step since primers are created based on the gene sequence (mRNA), thus the antisense strand must be duplicated for PCR to be successful. The reaction was then deactivated by heating the tubes at 70 °C for 15 min. Samples were stored at -20 °C.

2.2.6. Quantitative PCR

cytGPDH mRNA levels were quantified using SYBR Green I dye chemistry with normalization to 18S ribosomal RNA using a commercially available TaqMan assay and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Target amplifica-

tion through qPCR was performed in a 25 µL reaction composed of 1 µL forward and reverse (1 mM) primers (see Section 2.2.7.), 12.5 µL of 2X SYBR Green (Applied Biosystems, Foster City, CA), 1 µL cDNA, and water to achieve volume. Expression of target genes was normalized to 18S ribosomal RNA with Eukaryotic 18S rRNA Endogenous Control (VIC / MGB Probe, Primer Limited) (Applied Biosystems, Foster City, CA). 18S PCR amplification was performed in a separate 25 µL reaction using 0.4 ng of cDNA, 1X probe/primer mix and 1X TaqMan Universal PCR Master Mix, with AmpErase UNG (Applied Biosystems, Foster City, CA). The real time polymerase chain reactions were as follows: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, followed by 60 °C for 1 min. All samples were tested in duplicate on each plate. The fluorescence threshold cycle (C_T) was determined using the Applied Biosystems 7300 PCR Detection System SDS software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems, Foster City, CA). The relative starting quantity (RQ) of each transcript was determined with this software using the $2^{-\Delta\Delta CT}$ relative quantification method and assuming 100% amplification efficiencies (Livak and Schmittgen, 2001). The individual with the lowest normalized expression (mRNA) level was set as the calibrator sample (assigned a value = 1). This ensures that there are no mRNA expression values less than 1, which makes for a more simplistic statistical analysis. Gene expression data are presented as mean RQ (± standard error) relative to the calibrator.

A dilution series was performed for the 18S TaqMan assay for RNA extracted from rainbow smelt held at warm and cold temperatures. The 18S was found to be an effective

normalizer in this species (Hall, unpublished). For transcript studies the 18S normalizer TaqMan assays used 0.4 ng cDNA in each 25 µL reaction.

2.2.7. cytGPDH specific qPCR elements

The full length cDNA for rainbow smelt cytGPDH was cloned by Ewart et al. (2001). The cytGPDH gene (Accession # AY024368, 1096 bp) shows 87% identity to *Salmo salar* with an e-value = $2e^{-178}$. Primers derived from this gene sequence (forward: 5' TTCGTACAGAATGGCAGCAC 3'; reverse: 5' ACATTCCCACTGCATCCTTC 3') were used to amplify the 102 bp cytGPDH gene fragment with a melting temperature of 79 °C. Primer quality was assessed (dissociation curve analysis) to ensure single product amplification, absence of primer dimers in no template controls, and that amplification efficiency was between 90 and 110%. Amplification efficiency was calculated with hepatocytes isolated from warm- and cold-acclimated rainbow smelt (Hall, unpublished). For cytGPDH transcript studies 10 ng of cDNA was added to each 25 µL reaction.

2.2.8. Statistics

Within each group, values obtained at the various sampling dates were compared by ANOVA followed by Tukey's HSD post-hoc tests. t-tests were performed to compare warm- and cold-acclimated fish at each sampling date. In all cases $p<0.05$ was considered to be the level of statistical significance.

2.3. Results

2.3.1. GPDH tissue distribution

The plasma glycerol concentration of fish used in the tissue distribution of cytGPDH activity is shown in Figure 2.2. Glycerol concentrations were more than 100-fold significantly higher in cold-acclimated rainbow smelt than warm-acclimated rainbow smelt, capelin, or Atlantic salmon.

Studies detailing the activity of cytGPDH in multiple tissues, species, and conditions identified some key differences. cytGPDH liver activity was significantly higher in rainbow smelt than in the other species and was significantly higher in cold-acclimated rainbow smelt than in warm-acclimated fish (Figure 2.3A). Hepatic cytGPDH activity in cold-acclimated rainbow smelt approached 200 μmol/min • g tissue, whereas the activity in Atlantic salmon and capelin approached 10 and 25 μmol/min • g tissue, respectively.

White muscle cytGPDH activity in cold-acclimated rainbow smelt was significantly higher than in warm-acclimated rainbow smelt (11.99±1.00 versus 6.75±0.95 μmol/min • g tissue), but significantly lower than in warm- or cold-acclimated Atlantic salmon (19.83±1.26 and 21.12±1.77 μmol/min • g tissue respectively) (Figure 2.3B). Capelin muscle cytGPDH activity was the lowest of all species and conditions at 3.42±0.41 μmol/min • g tissue.

Heart, brain, and kidney cytGPDH activities are shown in Table 2.1. The two independent sampling dates for rainbow smelt and Atlantic salmon were not significantly different and were pooled for clarity. cytGPDH activity in brain and heart were, except for a few cases, similar with respect to species or acclimation condition. The only significant differences were in heart where activity was higher in the capelin as compared to cold-acclimated

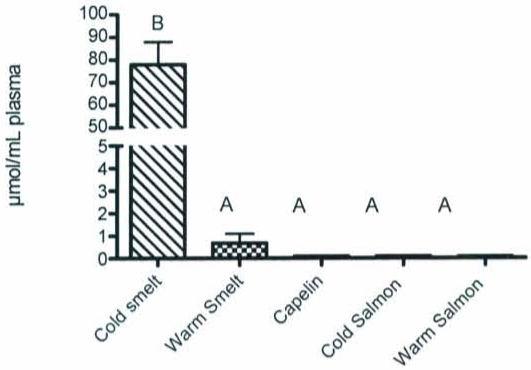

Figure 2.2: Plasma glycerol levels of cold- and warm-acclimated rainbow smelt and Atlantic salmon and capelin (8 °C). Different letters above the bars indicate significant difference (ANOVA: $p<0.05$) between sampling groups. Values are means ± SEM. N = 14 for rainbow smelt, 8 for capelin and 12 for Atlantic salmon.

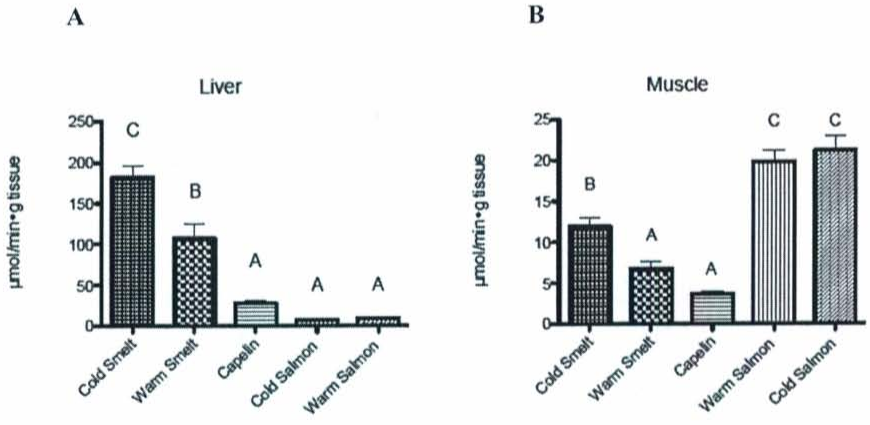

Figure 2.3: cytGPDH activity measured in cold- and warm-acclimated rainbow smelt and Atlantic salmon, and capelin liver (A) and white muscle (B). Different letters above bars indicate significant difference (ANOVA: $p<0.05$) between sample group. Values are means ± SEM. N = 14 for rainbow smelt, 8 for capelin and 12 for Atlantic salmon.

Atlantic salmon, and in brain where the activity was higher in cold- versus warm-acclimated Atlantic salmon. Kidney cytGPDH activity in cold- and warm-acclimated rainbow smelt and capelin were similar. However, the cytGPDH activity in the kidney of Atlantic salmon was significantly lower than the other species, irrespective of acclimation temperature.

Table 2.1: cytGPDH activity for heart, brain and kidney of cold- and warm-acclimated rainbow smelt and Atlantic salmon and capelin. Activity is expressed as μmol/min·g tissue. Values are means ± SEM with the N value in parenthesis. Different letters following values indicate significant differences ($p<0.05$) between groups within a tissue.

Fish	*Heart*	*Brain*	*Kidney*
Cold-Acclimated Rainbow smelt	2.97±0.37 (14) AB	0.94±0.14 (14) AB	7.09±0.75 (14) B
Warm-Acclimated Rainbow smelt	2.19±0.41 (14) AB	0.78±0.09 (14) AB	6.14±0.99 (14) B
Capelin	2.03±0.22 (8) B	1.31±0.10 (8) AB	6.66±0.62 (8) B
Cold-Acclimated Atlantic salmon	1.07±0.19 (12) A	0.51±0.07 (12) A	0.77±0.11 (11) A
Warm-Acclimated Atlantic salmon	2.21±0.52 (12) AB	1.50±0.35 (12) B	1.89±0.58 (12) A

2.3.2. cytGPDH isozyme

Analysis of the cellulose acetate gels showed four cytGPDH isoform bands in the liver (Figure 2.4a) and two in the white muscle (Figure 2.4b). The number and presence of rainbow smelt cytGPDH isozymes was independent of acclimation temperature.

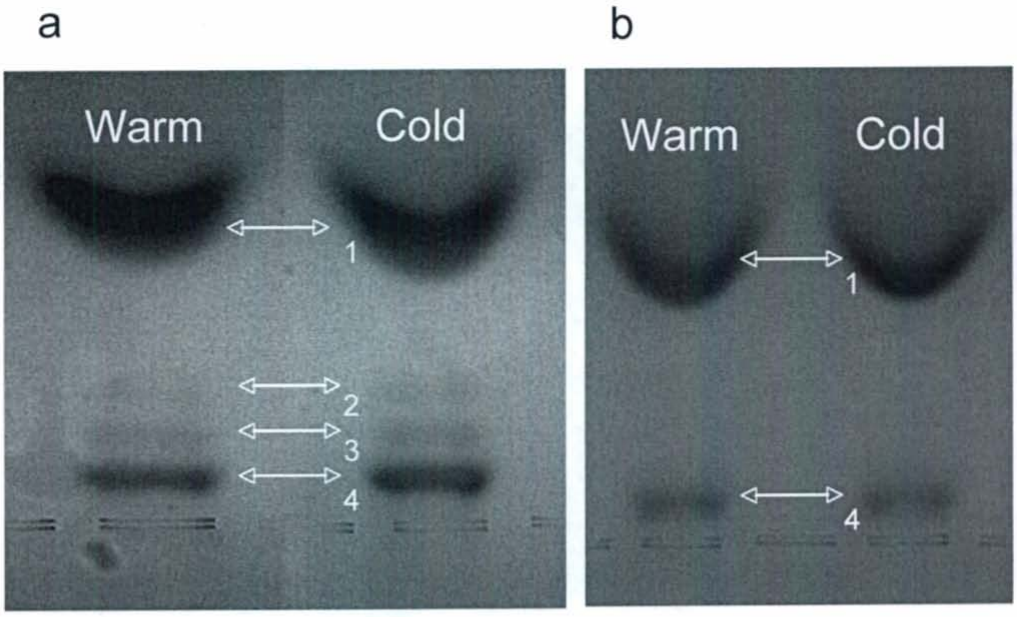

Figure 2.4: Cellulose acetate gels stained specific for partially purified cytGPDH from cold- and warm-acclimated rainbow smelt liver (a), and muscle (b). Bands indicate presence of a GPDH isoform through activity at certain points on the gel. Four isoforms were present in the liver (a) and 2 isoforms were found in white muscle (b).

2.3.3. Seasonal cytGPDH expression

Plasma glycerol concentrations for rainbow smelt used in the cytGPDH expression analysis study are shown in Figure 2.5. Glycerol increased to maximal levels by March 12, 2009 with glycerol concentrations reaching 128.2 +/- 9.38 mM. Glycerol concentrations dropped significantly after this date reaching basal levels (0.36 +/- 0.1 mM) by the final sampling point (May 4, 2009). The glycerol level in warm-acclimated rainbow smelt bordered the levels of detection throughout the study, demonstrating that they were not producing excess amounts. Since warm-acclimated rainbow smelt were not producing

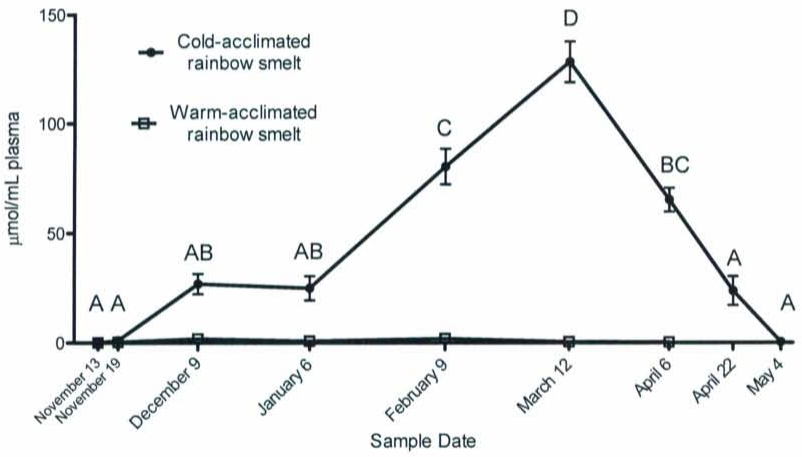

Figure 2.5: Plasma glycerol levels in warm- and cold-acclimated rainbow smelt from November 2008 to May 2009. Data points are means, +/- SEM. N = 4 for November 13, N = 8 for November 19 to February 9, all other sampling points N = 7. Letters indicate significant groupings for cold-acclimated rainbow smelt data points. Sampling dates sharing a letter are statistically similar at $p>0.05$.

glycerol, it is assumed that the observed specific activity and expression level of cytGPDH were due to factors other than glyceroneogenesis.

qPCR analysis of liver cytGPDH expression showed different seasonal profiles between the cold- and warm-acclimated rainbow smelt groups (Figure 2.6). Expression levels of cytGPDH in cold-acclimated rainbow smelt increased to maximal values in December (December 9, 2009) and slowly declined to baseline values by March 12, 2009 and through to May 4, 2009. In contrast, the warm-acclimated rainbow smelt showed no variation throughout the season. The differential regulation of cytGPDH between the two acclimation conditions is during the initiation of glycerol accumulation.

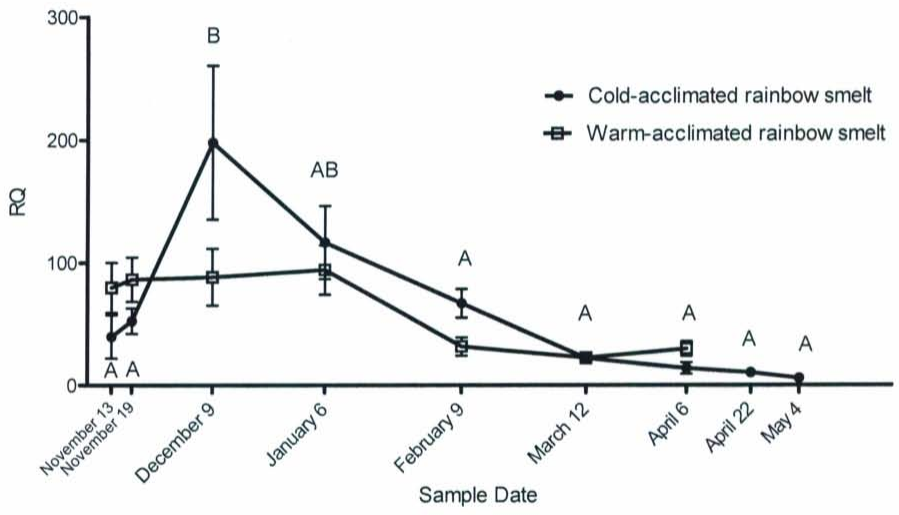

Figure 2.6: The relative quantity (RQ) of liver cytGPDH expression throughout the season, in warm- and cold-acclimated rainbow smelt. RQ values are means, +/- SEM. N = 4 for November - 13, N = 8 for November 19 to February 9, all other N = 7. Letters denote a significant difference between cold-acclimated data points. Sampling dates with a common letter are statistically similar at $p>0.05$.

2.4. Discussion

2.4.1. cytGPDH tissue distribution

The tissue distribution of cytGPDH activity showed both similarities and differences among the three teleost species. Rainbow smelt hepatic tissue had a significantly higher activity than measured in capelin or Atlantic salmon. This was expected given the non-glycerol accumulating nature of capelin and Atlantic salmon. The rainbow smelt appear to be poised for glycerol production given the high *in vitro* activity measured in the warm-acclimated individuals that are not producing glycerol. Furthermore, it is possible that high liver cytGPDH in the warm-acclimated rainbow smelt is a result of those indi-

viduals becoming gravid due to the constant exposure to higher than normal temperatures as previously suggested by Lewis et al. (2004). The activity of liver cytGPDH in rainbow smelt was substantially higher than all other tissues, species, or conditions tested in this study. However, it should be noted that the capelin and Atlantic salmon were likely more stressed than the rainbow smelt due to the sampling conditions. But it was assumed that the stress effects were minimal in these species and are thus considered negligible within the scope of this experiment.

The cytGPDH activity in white muscle revealed that capelin and warm-acclimated rainbow smelt had the lowest *in vitro* activity among the conditions and species analyzed. However, the cytGPDH activity in cold-acclimated rainbow smelt muscle was significantly higher (~2-fold) than in warm-acclimated individuals. Although, *in vitro* cytGPDH activity in muscle was significantly lower than measured in liver when expressed on a per gm basis, muscle could still be important for glycerol production at the whole animal level. The rainbow smelt hepato-somatic index is ~1.5 – 2% compared to muscle, which is estimated to be about 72% (>30-fold higher). Therefore, the cumulative action of muscle cytGPDH could make up a significant portion of accumulated glycerol. This contention remains to be tested. Muscle cytGPDH activity in rainbow smelt is significantly lower than in Atlantic salmon, a non-glycerol producing species. This phenomenon could be explained by the role of cytGPDH in triglyceride synthesis. Nutritional databases (for example <http://www.nutritiondata.com/>) reveal Atlantic salmon has 88% more total fat than rainbow smelt muscle. A high level of triglyceride synthesis would create demand for G3P which could be related to high *in vitro* cytGPDH activity in muscle.

No other tissue (brain, kidney, heart) emerged as a potential site of glycerol production in the rainbow smelt. However, all rainbow smelt tissues have higher glycerol concentrations in response to cold-acclimation (Raymond and Hassel 2000, Driedzic and Short 2007). The liver is implicated as the main source of glycerol production as other tissues, except potentially the muscle, have cytGPDH activity that is >20-fold lower than activity in liver. An interesting finding was the low kidney activity of cytGPDH in the Atlantic salmon. Capelin and rainbow smelt had higher kidney cytGPDH activities (>3-fold) compared to the Atlantic salmon. The reason for the large differences in *in vitro* cytGPDH kidney activity are unknown.

No articles could be found detailing tissue distribution of cytGPDH activity in any other organism comparable to rainbow smelt. Insects are the most well studied glycerol accumulating model, but the general small size of insects makes extraction of tiny body components both tedious and difficult. The flesh fly (*Sarcophaga bullata*) uses its brain to coordinate a glycerol accumulating response but the brain itself was not responsible for glycerol production (Yoder et al. 2005). Coordination of whole body metabolism through the brain points to an endocrine mechanism. Hormonal control would make physiological sense in the glycerol producing animal as it can coordinate many different genes, proteins and metabolism to elicit a desired response. Such a study in rainbow smelt has not been performed but hormonal control is expected to play a role in the multidimensional glycerol response.

2.4.2. cytGPDH isozymes

The cytGPDH electrophoretic analysis shows that the rainbow smelt liver possesses four putative isozymes. Previous studies in other species have identified two loci A* and B*. It has been determined that these loci translate into three homodimers (A_2', A_2'', and B_2), and one heterodimer (AB) (Basaglia and Cucchi 1995). Thus, rainbow smelt appear to use similar mechanisms to that of other teleosts with two differentially expressed cytGPDH paralogues. In an effort to assign analogous names to rainbow smelt cytGPDH isozymes the visible bands were matched based on the electrophoretic mobility and compared with previous studies. Rainbow smelt cytGPDH bands 3 and 4 appear to represent the two A* homodimers (A_2', A_2'') found in other teleosts and bands 1 and 2 represent the B* homodimer and the AB heterodimer, respectively (Fisher et al. 1980; Basaglia and Cucchi, 1993). Moreover, four liver cytGPDH DNA sequences have been identified in rainbow smelt via Southern Blot analysis (Ewart, unpublished). cytGPDH isoform nomenclature has yet to be resolved in teleosts as the A* and B* loci are isozymes but the location of the two A* alleles (A' and A'') is unknown, and therefore could represent allozymes (found at the same loci on different chromosomes).

Differential isoform expression of cytGPDH could make the rainbow smelt liver particularly poised for glycerol production. Differential regulation of cytGPDH isoforms is common in numerous higher order animals in association with specific functions (see for example: Phillipp et al. 1979, Ostro and Fondy, 1977, Basaglia and Cucchi, 1995, Kang et al. 1999, Stroppa, 2008). However, the actual purpose of each of the differentially regulated cytGPDH isoforms has not been identified in these previous studies. Further work is re-

quired to tease out the genetic components of cytGPDH isozyme/allozyme expression in rainbow smelt.

Rainbow smelt cytGPDH isozyme studies in white muscle revealed the presence of only two isoforms. This could be the result of numerous factors and could explain the lower observed cytGPDH activity in the muscle compared to liver. Moreover, fewer expressed muscle isoforms could be due to variable levels of penetrance found between tissues since muscle possesses the DNA template required to produce all four cytGPDH isoforms. However, due to the qualitative nature of the zymograms used in this study, increases in protein abundance or absence of isoforms are not definitive of cytGPDH patterns in rainbow smelt.

2.4.3. GPDH expression

Analysis of cytGPDH expression showed a peak in December in cold-acclimated rainbow smelt, similar to the trend found by Liebscher et al. (2006). This spike was not found in warm-acclimated fish and therefore is assumed to be a cold response. This cold-acclimated December spike was followed by a steady decline that lasted until basal levels were reached in March. These data are consistent with previous cytGPDH activity and expression studies. Combining the current data, as well as, data from Liebscher et al. (2006) and Lewis et al. (2004), it is clear that a surge in cytGPDH expression followed by a surge of cytGPDH activity occurs at the onset of glycerol production (Figure 2.7).

This study also examined for the first time the seasonal profile of cytGPDH expression in rainbow smelt maintained at a constant warm temperature. The beginning of the

season was marked by sustained cytGPDH expression in the warm-acclimated individuals as opposed to the rapid elevation observed in the fish tracking the seasonal decrease in water temperature. Expression in these individuals did not vary statistically but a decrease was observed during the season between January 6 and February 9, 2009; after which expression was maintained at low levels. The differences in the response between the two groups emphasizes the concept that the cytGPDH expression is activated by low temperature in the rainbow smelt.

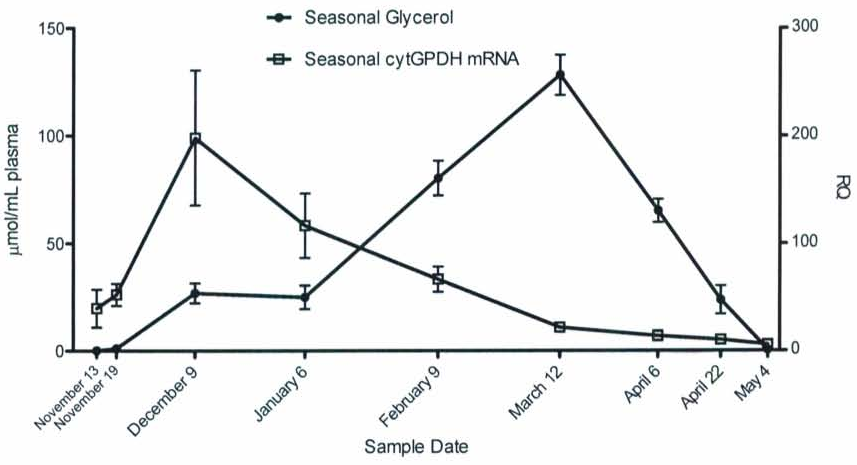

Figure 2.7: Plasma glycerol and cytGPDH expression level in cold-acclimated rainbow smelt throughout the winter season. Data points and descriptions are found in Figures 2.5 and 2.3.

2.5. Conclusions

This study supports the concept that the liver is poised as the primary location for glycerol production in cold-acclimated rainbow smelt. White muscle may also produce glycerol but this tissue does not contain the same (per gram) cytGPDH catalytic capacity as

liver, possibly due to fewer isozymes. The liver of rainbow smelt expresses four cytGPDH isozymes similar to other teleost species. The lack of quantitative data for the cytGPDH isozymes does not allow for the assertion that all rainbow smelt cytGPDH forms are equally transcribed, ergo no single cytGPDH form can be implicated as being responsible for the control of glycerol production. Quantitative analysis of cytGPDH mRNA levels shows increased cytGPDH expression when plasma glycerol levels are beginning to accumulate in the cold-acclimated individuals. The warm-acclimated group cytGPDH expression does not show an analogous peak, and thus the expression that occurs in this group is assumed to be independent of glycerol production.

3. FAD-linked glycerol 3-phosphate (mGPDH) plays a role in seasonal glycerol decrease in the rainbow smelt (*Osmerus mordax*)

3.1. Introduction

Rainbow smelt (*Osmerus mordax,* Mitchill, 1814) accumulate massive quantities of glycerol in response to cold temperatures with plasma concentrations, in some fish, reaching >400 mM (Raymond, 1997). The study of glycerol accumulation in rainbow smelt has been dominated by the investigation of physiological glycerol production (see for example: Driedzic et al. 2006, Driedzic et al. 1997, Raymond, 1995). In hepatocytes isolated from rainbow smelt, glycerol production can be induced by cold temperature alone. The glycerol in these cold-incubated cells is immediately derived from liver glycogen which is reflected by significant increases in glycogen phosphorylase activity (Clow et al. 2008). Other glycerol substrates recognized from whole animal studies include dietary glucose and protein (Walter et al. 2006). The concentration of plasma glycerol in the rainbow smelt reaches a maximum in March and decreases to more physiologically common teleost levels by May (Lewis et al. 2004; Chapter 2). This sharp and predictable glycerol decrease occurs while water temperature is still low, and thus it is not a function of warming water. Whole body glycerol is constantly lost through the skin and gills of the rainbow smelt at a rate of about 3.8 - 13.4% glycerol per day (Raymond, 1993), but in concert with the spring decrease in glycerol is an increase in liver glycogen, implying that a portion of the glycerol is retained (Driedzic and Short, 2007). Furthermore, the exclusive loss of glycerol through the body surface would represent an unnecessary waste of potential energy.

Glycerol catabolism must first occur through glycerol kinase, an enzyme that catalyzes the phosphorylation of glycerol to the three carbon alcohol glycerol 3-phosphate (G3P) (Figure 3.1). This enzyme is found in rainbow smelt liver and its *in vitro* activity increases late in the season when glycerol levels are returning to pre-winter concentrations (D. Ditlecadet, unpublished). Once G3P is formed there are two possible fates of this intermediate, G3P can be acylated into the triglyceride pool or be be converted to

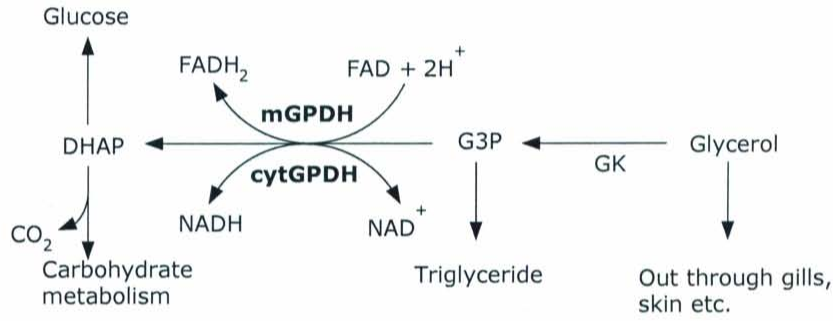

Figure 3.1: The possible routes of glycerol catabolism in the rainbow smelt. Excess glycerol can diffuse into the ocean through the body surface. Alternatively, it can be phosphorylated to G3P through catalysis by glycerol kinase. G3P can then be acylated into triacylglyceride or be oxidized into DHAP through cytGPDH or mGPDH. The DHAP can then join the glycolytic/glucogenic

dihydroxyacetone phosphate (DHAP). DHAP formation could occur through cytGPDH an enzyme that has been shown to be involved in glycerol accumulation (see for example: Lewis et al. 2004, Driedzic et al. 1998, Liesbscher et al. 2006). Alternatively, DHAP can be formed by mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH) an enzyme that catalyzes the formation of DHAP from G3P, yielding $FADH_2$ in the process. mGPDH is embedded in the outer face of the inner mitochondrial membrane (Klingenberg, 1970) and, together with cytGPDH, forms the glycerophosphate shuttle. The shuttle functions to pass reducing equivalents in the form of NADH from the cytosol into the inner membrane

through $FADH_2$ formation. Rainbow smelt mGPDH has not been measured but could be an essential component of the seasonal regulation of glycerol.

A seasonal study was employed to measure mGPDH activity and expression throughout the glycerol production and termination phases. Glycerol production has been previously shown to be dominant in the liver (Chapter 2) and therefore it followed that hepatic mitochondria were a good starting point to measure the mGPDH protein. The *in vitro* activity of hepatic mGPDH was analyzed along with *in vivo* expression levels in rainbow smelt exposed to seasonal water temperatures between November 2008 (~10 °C) and May 2009 (~2 °C).

3.2. Materials and Methods

3.2.1. Tissue sampling and harvest

Rainbow smelt used in this study were the same as those sampled in Chapter 2 for the assessment of cytGPDH expression. Rainbow smelt were collected from fresh water at Mount Arlington Heights, Placentia Bay by a seine net in November 2008. Fish were transported in sea water to the Ocean Sciences Centre, Memorial University, Logy Bay, NL and randomly sorted into two separate 3000 L tanks. One tank followed the ambient seasonal sea water temperature herein referred to as cold-acclimated. The second tank received flow through sea water heated to ~10 °C throughout the study, and this group is referred to as warm-acclimated. Fish were fed chopped herring 2-3 times per week with both tanks held on a natural photo-period with fluorescent lights set to an outdoor photocell. Temperatures of the two tanks were recorded (see Figure 2.1). Rainbow smelt were sam-

pled approximately every 30 days from November 2008 to May 2009, independent of sex or size. A sample of whole blood was extracted from the caudal vein with heparanized syringes and spun at 10,000 x g for 5 min to extract the plasma. Immediately after killing by a blow to the head ~100 mg of liver was flash frozen in liquid N_2 and stored at -80 °C. The remainder of the liver was placed on ice for immediate mitochondrial extraction.

3.2.2. Mitochondrial extraction

Liver mitochondria from rainbow smelt were extracted using a modified differential centrifugation technique based on methods from Ballantyne (1994). Whole livers were weighed and ground with a pestle at ~100 rpm in 9 volumes of ice cold incubation media (500 mM sucrose, 30 mM hepes, 5 mM EDTA, and 5 mM EGTA, pH 7.4). The homogenate was passed through cheesecloth into a centrifuge tube and spun for 10 min at 800 x g. The supernatant was again passed through cheesecloth and collected in a new centrifuge tube and spun at 9,000 x g for 10 min. The resulting supernatant was discarded and the mitochondrial pellet was resuspended by gentle pipetting into 10 mL of ice cold resuspension media (300 mM KCl, 30 mM Hepes, 10 mM K_2HPO_4, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4). Mitochondrial suspensions were spun at 9,500 x g for 10 min and the supernatant was discarded. The mitochondrial pellet was again resuspended in 9 volumes of ice cold resuspension media. Centrifugation steps took place in a refrigerated centrifuge that was pre-chilled to 1 °C.

3.2.3. mGPDH INT based activity assay

The activity of mGPDH was measured with a method adapted from Gardner (1974). 250 µL of mitochondrial suspension was added to 2 mL snap tubes along with 250 µL of iodoformazan (INT) buffer (4 mM INT, 1 mM KCN, 400 mM K_2PO_4, 0.1 mM FAD^+, 1 µM Antimycin A, pH 7.4). The reaction was initiated with 50 µL of 40 mM G3P (an enzyme saturated concentration), the tubes were centrifuged for ~5 sec and incubated in the dark for 30 min at room temperature. The reaction was terminated by the addition of 100 µL of acetic acid and 1 mL of ethyl acetate. Reaction tubes were vortexed for 15 seconds to separate the organic and non-organic phases. Absorbance of the upper organic phase was measured at 490 nm in an LKB Biochrom Novaspec spectrophotometer. Formation of formazan from INT was used to measure oxidation through FAD-linked GPDH. INT is reduced to formazan as FAD is reduced to $FADH_2$ through the action of mGPDH. Data are expressed as µmol/min • mg protein.

3.3.4. mGPDH qPCR primers

Total RNA extraction from liver, DNAse treatment, cDNA synthesis and qPCR methods are found in Chapter 2 (Sections 2.2.5 - 2.2.7).

The full length cDNA for rainbow smelt mGPDH was cloned by Hall and Driedzic (unpublished). The mGPDH gene (Accession # FJ797643, 2805 bp) has 79% identity to the *Xenopus tropicalis* mGPDH gene with an e-value = 0. Primers were derived from this gene sequence (forward: 5' TTGGCCAAGATGGCACATGT 3'; reverse: 5' CATGGCATACAACACCTCGCT 3', 95% efficiency) and used to amplify a 101 bp fragment with a

melting temperature of 80.5 °C. Primer quality was assessed (dissociation curve analysis) to ensure single product amplification, absence of primer dimers in no template controls, and an amplification efficiency between 90 and 110%. The amplification efficiency of these primers were calculated using hepatocytes from warm- and cold-acclimated rainbow smelt (Hall, unpublished). For the mGPDH gene 50 ng of cDNA was added per 25 μL qPCR reaction.

3.2.5. Protein concentration

Protein concentrations were determined with the Bradford method using the Coomassie Plus (Bradford) Protein Assay (Thermo Scientific, Rockford, IL). Briefly, 30 μL of mitochondria was added to 1.5 mL of Coomassie reagent, mixed, and the absorbance was read at 595 nm at room temperature. Concentrations were determined using a standard curve with the albumin standard provided by Thermo Scientific. In this assay, red pigments in the coomassie dye specifically bind to ionizable groups of proteins which stabilize blue pigments in the coomassie dye. The blue pigments absorb maximally at 595 nm, thus the absorbance is proportional to protein concentration in a sample.

3.2.6. Statistics

Within each group, values obtained at the various sampling dates were compared by ANOVA followed by Tukey's HSD post-hoc tests. Independent samples t-tests were performed to compare warm- and cold-acclimated fish at each sampling date. Regression

analysis was also performed to examine glycerol dependence on mGPDH activity and expression levels. In all cases $p<0.05$ was considered to be the level of statistical significance.

3.3. Results

3.3.1. Seasonal profile of FAD-linked GPDH activity

The seasonal mGPDH activity in cold-acclimated rainbow smelt was characterized by significant increases at the end of the season when compared with November samples. The highest level was observed in the final sample (May 4) with a maximal activity of 4.71 +/- 0.45 μmol/min • mg protein (Figure 3.2). This activity is 2.43-fold higher than the initial November 13, 2009 level.

The regression analysis performed during the unequivocal glycerol decrease phase (April 6 to May 4, 2009) showed a significant negative correlation between mGPDH activity in cold-acclimated rainbow smelt and plasma glycerol levels with a p-value of 0.003 (Figure 3.3). Data from March 12, 2009 was not included in this analysis since it cannot be stated with certainty that maximal glycerol levels were attained by that date.

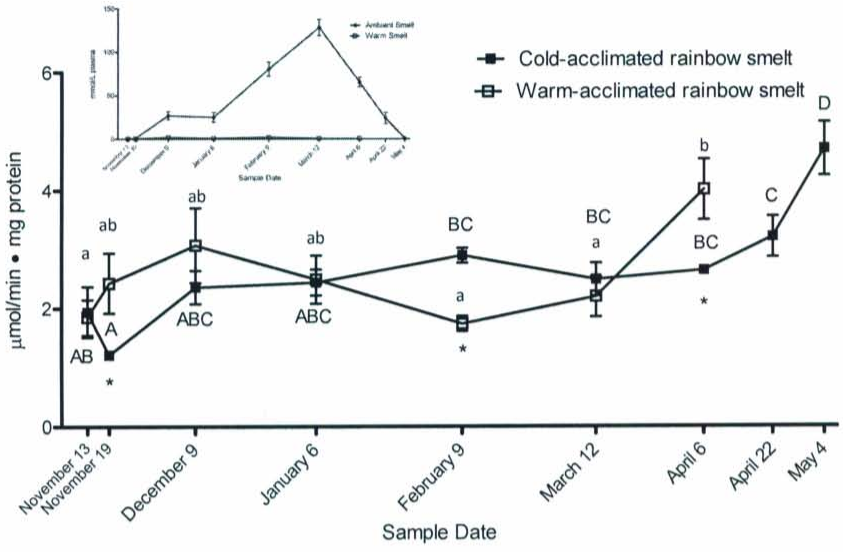

Figure 3.2: mGPDH activity throughout the season in cold- and warm-acclimated rainbow smelt. Values are means +/- SEM. N = 4 for November 13, N = 8 for November 19 to February 9, for all others N = 7. Activities are presented as µmol/min• mg protein. Lower case letters indicate Tukey's HSD groupings for warm-acclimated fish and upper case represent cold-acclimated fish. * Denotes a significance between cold- and warm-acclimated rainbow smelt. The plasma glycerol profile is shown within the graph (Chapter 2).

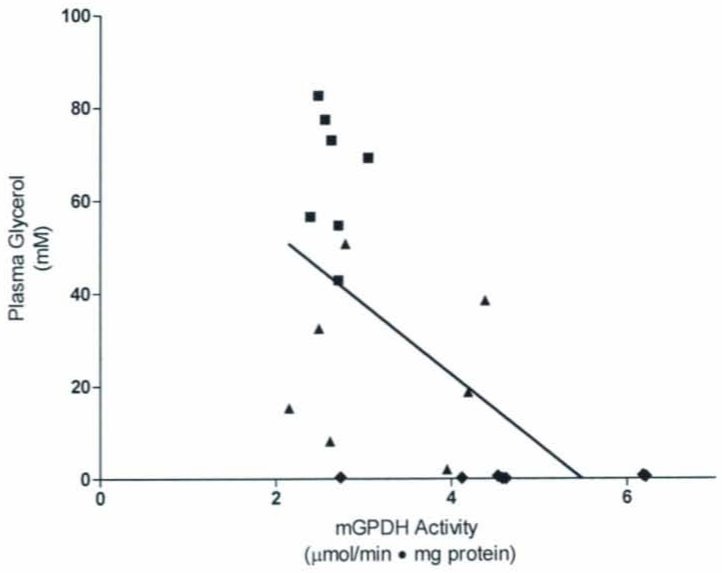

Figure 3.3: Regression analysis relating plasma glycerol concentration to the activity of liver mGPDH in cold-acclimated rainbow smelt. The graph represents mGPDH activity during the glycerol decrease phase. This relationship has a p-value of 0.003 and an R^2 value of 0.4. The squares, triangles and diamonds represent April 6, 22, and May 4 sample dates respectively.

The mGPDH activity in the warm-acclimated rainbow smelt showed the final measure in April to be significantly higher than the first sample (Nov 13). The final sample (April 6) ended as maximum with an activity of 4.02 +/- 0.51μmol/min • mg protein. Sampling for the warm-acclimated individuals was not possible past the April 6 date due to mortalities.

3.3.2. Seasonal profile of FAD-linked GPDH expression

Levels of mGPDH expression in cold- and warm-acclimated rainbow smelt did not vary over the sampling period (Figure 3.4). However, in cold-acclimated rainbow smelt the

average value in April 6, 2009, when plasma glycerol was clearly declining, was 4-fold higher than November levels. Moreover, significant differences were found between the cold- and warm-acclimated samples in the December 9, and February 9 samples. The mean April 6 cold-acclimated smelt sample was also higher than the level in warm-acclimated fish but, it did not quite reach statistical significance ($p = 0.06$).

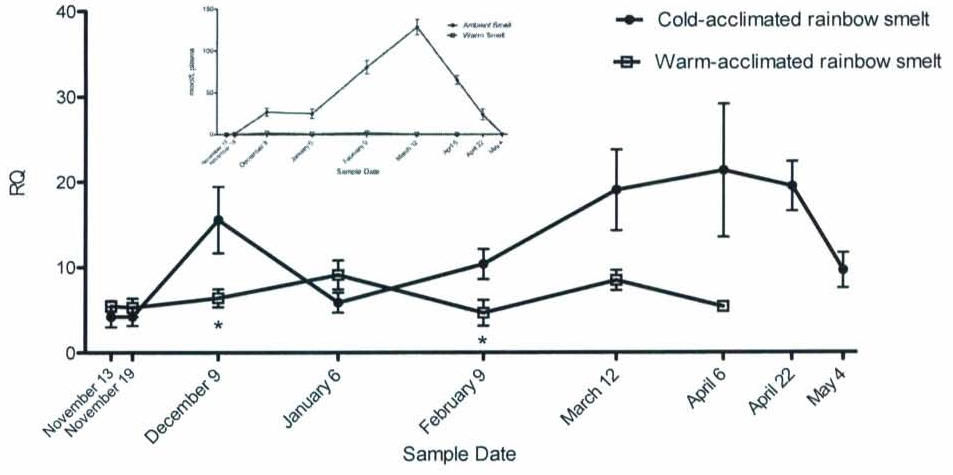

Figure 3.4: The mGPDH mRNA abundance in cold- and warm-acclimated rainbow smelt sampled throughout the winter season. Relative quantity (RQ) values are means +/- SEM. N = 4 for November 13, N = 8 for November 19 to February 9, for all others N = 7. * indicates a significant difference between cold- and warm-acclimated rainbow smelt ($p<0.05$).

3.4. Discussion

3.4.1. Hepatic mGPDH protein may play a role in glycerol decrease

Plasma glycerol concentration has been measured for more than a decade to evaluate the status of glycerol production in cold-acclimated rainbow smelt (see for example: Driedzic et al. 1998, Treberg et al. 2002b, Clow et al. 2008). The cold-acclimated fish used

in this study showed the expected pattern with glycerol levels achieving a maximum in mid-late winter (March 12, 2009) and falling to baseline levels in the early spring (May 4, 2009). This annual glycerol increase is activated by temperature alone (Clow et al. 2008), with cytGPDH and phosphoenol pyruvate carboxykinase (PEPCK) considered key regulatory sites (Lewis et al. 2004, Liebscher et al. 2006). However, to date there are no proposed mechanisms for the decrease in glycerol other than direct loss through the body into the water (Raymond, 1993).

The activity of the mGPDH enzyme measured throughout the season in the ambient held fish showed significant changes during the natural seasonal glycerol increase and decrease period. The mGPDH activity in cold-acclimated rainbow smelt was minimal in the November 19, 2009 samples. At this early time point plasma glycerol concentrations are still at a non-accumulating level. Throughout the glycerol accumulation phase (December to March) mean mGPDH activity did not change significantly in the cold-acclimated individuals. Germane to the hepatic mGPDH activity study are changes observed during the glycerol decline. Plasma glycerol levels (Figure 2.1) rapidly decreased after the March sample (March 12, 2009) eventually falling to basal levels by May (May 2, 2009). During the period of unequivocal glycerol decline (ie. April 6 to May 4, 2009) mGPDH activity surged to maximal levels at the final measurements,

Based on the assumption that plasma glycerol level reflects concentration of G3P in the liver, it is paradoxical that the activity of mGPDH should increase as the substrate decreases. The reason for this may be due to the fixed position of mGPDH within the inner mitochondrial membrane, as opposed to an enzyme that has mobility within the cytosol. It

may be that the only way to enhance chances of an enzyme-substrate interaction is through the increase of mGPDH protein available for catalysis at the inner surface of mitochondrion.

I propose that high levels of mGPDH protein are available to ensure that all remaining glycerol is catabolized. In support of this contention is the significant relationship between the plasma glycerol concentration and mGPDH activity, and the observed surge in catalytic capacity at the end of the glycerol decline. Furthermore, a simple calculation (assuming a uniform whole body glycerol concentration) shows that a 100 gm individual must clear ~5500 μmol of glycerol from April 6 to May 4, 2009. If all glycerol is metabolized in the liver and an average liver mass is ~2.5 gm that would require a constant mGPDH activity of 0.05 μmol/min•gm liver over the ~28 days. A typical liver contains about ~0.5% mitochondrial protein (~0.5 mg in a 1 gm liver) thus the required rate to fully clear glycerol over the 28 day period would approximate to 0.1 μmol/min•mg mitochondrial protein. Therefore, a mGPDH activity of 3.22 μmol/min•mg mitochondrial protein is more than adequate to catabolize whole body glycerol. Fluxing glycerol derived carbon through mGPDH is a more economical path than losing glycerol to the environment as it generates ATP and allows glycerol to be sequestered as glycogen.

The maximal mGPDH activity in warm-acclimated rainbow smelt liver was on April 6, 2009 with a significantly higher activity than observed on November 13; February 9 and March 12. The final data point must be treated with caution due to a technical error. The final mGPDH activity measurement was calculated using averaged protein concentrations from previous mitochondrial extractions and was assumed to be similar to the April 6,

2009 samples. Based on available data the possibility that mGPDH activity also increases late in the season, in fish held at warm temperature, cannot be ruled out. Indeed, it may be that the signal for glycerol decline is dependent on photoperiod as opposed to temperature triggered (Driedzic and Short, 2007). Regardless, this does not detract from the concept that elevated levels of mGPDH in fish exposed to natural seasonal water temperatures are critical in the final step in glycerol clearance.

Given mGPDHs position at the junction of several metabolic pathways some seasonal regulation of activity is not surprising. Minor changes in mGPDH activity in the warm-acclimated rainbow smelt are to be expected due to mGPDH's position at the interface between glycolysis and respiration. Animals acclimated to warm conditions can become gravid in March/April and vitellogenesis results in an alteration of overall metabolism in teleosts (Salem et al. 2006). Alternatively, an increase in mGPDH activity in both the warm- and cold-acclimated rainbow smelt could point to a seasonal regulatory mechanisms based on photoperiod that is independent of temperature.

3.4.2. Relationship between mGPDH expression, activity and plasma glycerol concentration

qPCR studies followed the expression level of the mGPDH amplicon throughout the glycerol cycle in the cold-acclimated rainbow smelt. No statistical significance was found throughout the season, perhaps due to high SEM values at some points. High variability is expected since all the individuals are dealing with major shifts in metabolism, in-

dependently, and therefore at any given time each individual may be at a different state depending on their metabolism, size, and overall health.

However, a trend was observed in the cold-acclimated individuals with an increase in mean values from March 12, 2009 until April 22, 2009. The February increase in average value is prior to the peak plasma glycerol concentration found in March, thus possibly preparing the cold-acclimated individuals for the glycerol decrease through the supply of mGPDH protein. Furthermore, treating the data as phases reveals the relative mGPDH expression from November 13 to February 9 (ie. Glycerol accumulation) is 8.27 ± 1.25 ($N = 34$); whereas, during the glycerol decline phase from April 6 to May 4 it is 16.83 ± 3.06, a significantly higher value. Again the March value is omitted as it cannot be determined if it is part of glycerol increase, or decrease.

Together, the data imply a mechanism whereby expression is maintained at a high level throughout the glycerol decrease phase to provide enough translated mGPDH protein for glycerol catabolism. In rats, mGPDH activity is governed by T3 hormones (Rauchova et al. 2004); however, in rainbow smelt there is currently no information regarding mGPDH activation by thyroid hormones as is found in other species. With mGPDH playing such an important role regarding the overall metabolic status in other organisms, it is tenable that such a situation also exists in the rainbow smelt. The present study suggests that mGPDH plays a role in the glycerol decrease process and given the hormonal stimulation of mGPDH in other organisms a hormonal mechanism is also speculated in the rainbow smelt (Gambert 2005, Gong et al. 1998; Lee and Lardy, 1965; Muller and Seitz, 1994, Urecelay et al. 2000).

3.5. Conclusions

This study marks the first time that mGPDH has been analyzed in rainbow smelt, as well as, the first time it has been implicated as a component of glycerol regulation in this species. The data presented implies a significant physiological role for mGPDH in metabolizing glycerol. Regression analysis supports this as declining plasma glycerol concentrations show a significant dependence on mGPDH activity. The levels of mGPDH expression are higher during glycerol decline than prior to glycerol accumulation and high expression levels are consistent with higher enzyme activity. High mGPDH activity at the end of the season potentially leads to the capture of glycerol as opposed to losing it to the environment.

4. Summary

The study of any biochemical process, such as glycerol production in rainbow smelt (*Osmerus mordax,* Mitchill, 1814) requires comparison of the physiological components prior to analyzing the process as a synergistic whole. This study analyzed essential components of glycerol production in the rainbow smelt. NAD-linked glycerol 3-phosphate dehydrogenase (cytGPDH) is involved with the onset of glycerol production and FAD-linked glycerol 3-phosphate dehydrogenase (mGPDH) is involved with declining glycerol levels.

The detailed analysis of cytGPDH found in Chapter 2 showed that cytGPDH in rainbow smelt is most active in liver. This is based on a significantly higher hepatic cytGPDH activity in glycerol producing rainbow smelt compared to fish that were not accumulating glycerol. Moreover, hepatic cytGPDH activity was higher than all other tissues, and species studied. Despite strong evidence to support the liver as the sole site of glycerol synthesis, white muscle, a much larger tissue, also had significantly higher cytGPDH activity in glycerol producing rainbow smelt compared to warm-acclimated individuals. Muscle, however, in comparative studies had lower cytGPDH activity than Atlantic salmon which is a non-glycerol accumulating teleost species. Furthermore, isozyme studies showed four putative isozymes in rainbow smelt liver and only two in the muscle. Future studies regarding cytGPDH protein should focus on the genetic nature of these isozymes in the two tissues, given that Southern blot analysis has yielded four different cytGPDH genes in liver (Ewart, unpublished). Experiments analyzing the differential regulation of the four transcripts, as well as western blots, would further enhance our understanding of glycerol regulation.

Gene expression studies of the GPDH proteins show similar profiles during periods of glycerol increase. cytGPDH transcript levels increase when the rainbow smelt are actively synthesizing glycerol and cytGPDH activity and plasma glycerol have a significant positive relationship (Lewis et al. 2004). It is proposed that during the initiation of glycerol accumulation the increased expression of both cytGPDH and mGPDH is due to enhanced regulation of the glycerophosphate shuttle, but this cannot be confirmed. However, during the decline in plasma glycerol levels, the mGPDH expression patterns are opposite of cytGPDH (Figure 4.1). Moreover, mGPDH expression, prior to glycerol production is significantly less than during glycerol decrease. Furthermore, the activity of hepatic mGPDH significantly increased during plasma glycerol decline and is associated with declining glycerol concentration.

Overall, this study demonstrated a significant dependency of rainbow smelt glycerol accumulation and decline upon the action of the two dehydrogenase enzymes. Both proteins play significant roles in the level of plasma glycerol at some point during the season. Because of the physiological roles the two enzymes play regarding overall metabolism, their involvement in glycerol regulation is not surprising. Hibernation studies have shown the two enzymes to be actively controlled by various hormones, especially those of the thyroid gland, as well as glucocorticoids (Berrada et al. 2000). Further studies regarding the activation of these two enzymes in rainbow smelt should focus on hormonal changes that could be responsible for invoking a shift from glycerol accumulation to clearance.

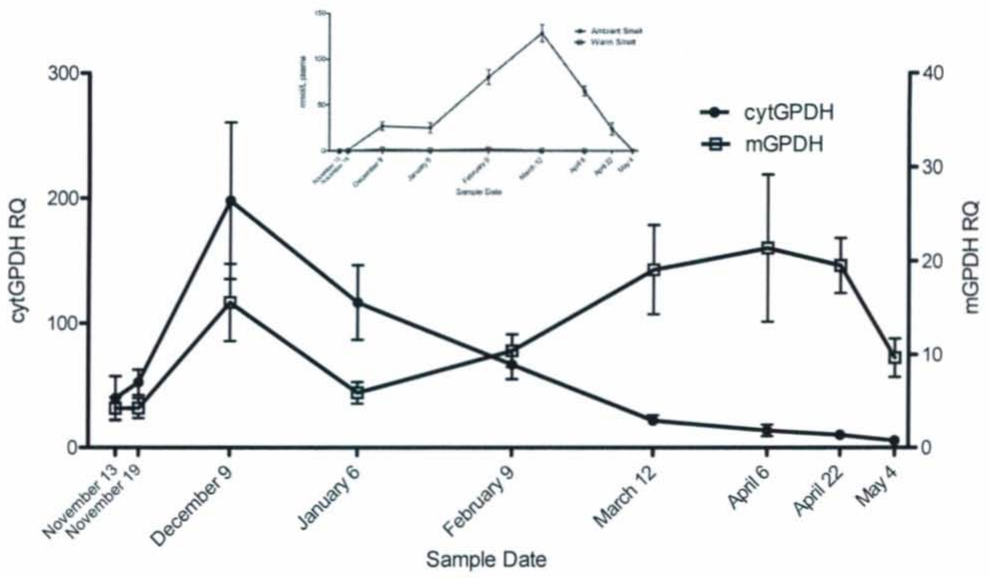

Figure 4.1: Differential expression of the two GPDH (cytosolic and mitochondrial) genes in the cold-acclimated rainbow smelt over the season. The plasma glycerol concentration is included for reference. The expression of both genes increases during glycerol production but mGPDH peaks a second time during glycerol loss.

5. Literature Cited

Ballantyne, J.S. *Fish Mitochondria.* From: *Biochemistry and molecular biology of fishes. vol.* 3. Ed. Hochachka and Mommsen. 1994, Elsevier Science B.

Basaglia, F., Cucchi, C. Effects of benzidine on the electrophoretic behaviour of some enzymes in *Ictalurus* species (Ictaluridae, Teleostei). *Comp. Biochem. Physiol.* 104C, 225-232. 1993.

Basaglia, F., Cucchi, C. Variations induced by phenylhydrazine on glycerol 3-phosphate dehydrogenase isozymes in black bullhead, *Ictalurus melas* (Silurifmes, Ictaluridae). *Cytobios.* 82, 139-150. 1995.

Bewley, G.C., Cook, J.L. Molecular structure, developmental regulation, and evolution of the gene encoding glycerol 3-phosphate dehydrogenase isozymes in *Drosophila melanogaster. Isozyme* 3: 341–374. 1990.

Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72,* 248-254. 1976.

Canestro, C., Yokoi, H., Postlethwait, J.H. Evolutionary developmental biology and genomics. *Nat. Rev. Genetics*. 8, 932-942. 2007.

Clow, K.A., Ewart, K.V., Driedzic, W.R. Low temperature directly activiates the initial glycerol antifreeze response in isolated rainbow smelt (*Osmerus mordax*) liver cells. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R961-970. 2008.

Driedzic, W.R., Clow, K.A., Short, C.E., Ewart, K.V. Glycerol production in the rainbow smelt (*Osmerus mordax*) may be triggered by low temperature alone and is associated with the activation of the glycerol 3-phosphate dehydrogenase and glycerol 3-phosphatase. *J. Exp. Biol.* 209, 1016-1023. 2006.

Driedzic, W.R., Short, C.E. Relationship between food availability, glycerol, and glycogen levels in low-temperature challenged rainbow smelt *Osmerus mordax. J. Exp. Biol.* 210, 2866-2872. 2007.

Driedzic, W.R., West, J.L., Sephton, D.H., Raymond, J.A. Enzyme activity levels associated with the production of glycerol as an antifreeze in liver of rainbow smelt (*Osmerus mordax*). *Fish Physiol. Biochem.* 18, 125-134. 1998.

Ewart, K.V., Fletcher, G.L. Isolation and characterization of antifreeze proteins from smelt (*Osmerus mordax*) and atlantic herring (*Clupea harengus harengus*). *Can. J. Zool.* 68, 1652-1658. 1990.

Ewart, K.V., Richards, R.C., Driedzic, W.R. Cloning of glycerol 3-phosphate dehydrogenase cDNA from two fish species and effect of temperature on enzyme expression in rainbow smelt (*Osmerus mordax*). *Comp. Biochem. Physiol. B.* 128, 401-412. 2001.

Fisher, S.E., Shaklee, J.B., Ferris, S.D., Whitt, G.S. Evolution of five multilocus isozymes in the chordates. *Genetica* 52-53, 73-85. 1980.

Fletcher, G.L., Kao, M.H., Dempson, J.B. Lethal freezing temperatures of arctic char and other salmonids in the presence of ice. *Aquac.* 71, 369-378. 1988.

Gambert, S., Helies-Toussaint, C., Grynberg, A. Regulation of intermediary metabolism in rat cardiac myocyte by extracellular glycerol. *Biochim. Biophys. Acta.* 1736, 152-162. 2005.

Gardner, R.S. A sensitive colorimetric assay for mitochondrial ∝-glycerophosphate dehydrogenase. *Anal. Bioc.* 59, 272-276. 1974.

Gong, D.W., Bi, S., Weintraub, B.D., Reitman, M. Rat mitochondrial glycerol 3-phosphate dehydrogenase gene: multiple promoters, high levels in brown adipose tissue, and tissue-specific regulation by thyroid hormone. *DNA Cell. Biol.* 17, 301-309. 1998.

Joanisse, D.R., Storey, K.B., Enzyme activity profiles in an overwintering population of freeze-tolerant larvae of the gall fly, *Eurosta solidaginis. J. Comp. Physiol. B.* 164, 247-255. 1994.

Kang, S.J., Park, J.Y., Park K.S. Effect of dietary carbohydrates on the expression of GPDH isozymes in *Drosophila melanogaster*. *Korean J. Genetics.* 21, 19-28. 1999.

Klingenberg, M. Localization of the glycerol-phosphate dehydrogenase in the outer phase of the mitochondrial inner membrane. *Eur. J. Biochem.* 13, 247-252. 1970.

Knight, C.A., Devries, A.L. Effects of a polymeric, nonequilibrium 'antifreeze' upon ice growth from water. *J. Crys. Grow.* 143, 301-310. 1994.

Lee, Y.P., Lardy, A. Influence of thyroid hormones on L-alpha glycerophosphate dehydrogenase and other dehydrogenases in various organs of the rat. *J. Biol. Chem.* 240, 1427-1436. 1965.

Lewis, J.M., Ewart, K.V., Driedzic, W.R. Freeze resistance in rainbow smelt (*Osmerus mordax*): seasonal pattern of glycerol and antifreeze protein levels and liver enzyme activity associated with glycerol production. *Physiol. Biochem. Zool.* 77, 415-422. 2004.

Liebscher, R.S., Richards, R.C., Lewis, J.M., Short, C.E., Muise, D.M., Driedzic, W.R., Ewart, K.V. Seasonal freeze resistance of rainbow smelt (*Osmerus mordax*) is generated by differential expression of glycerol 3-phosphate dehydrogenase, phosphoenolpyruvate carboxykinase, and antifreeze protein genes. *Physiol. Biochem. Zool.* 79, 411-423. 2006.

Livak, K.J.and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408. 2001.

MacDonald, M.J., Brown, L.J. Calcium activation of mitochondrial glycerol phosphate dehydrogenase restudied. *Arch. Biochem. Biophys.* 326(1) 79-84. 1996.

Muller, S., Seitz, H.J. Cloning of a cDNA for the FAD-linked mitochondrial glycerol 3-phosphate dehydrogenase from rat liver and its regulation by thyroid hormones. *Proc. Natl. Acad. Sci. USA* 91, 10581-10585. 1994.

Ostro, M.J., Fondy, T.P. Isolation and charcterization of multiple molecular forms of cytosolic NAD-linked glycerol 3-phosphate dehydrogenase from normal and neoplastic rabbit tissues. *J. Biol. Chem.* 252, 1977.

Park, K.S., Bae, Y.J., Yeau, S.H., Kang, S.J. Cloning and sequence analysis of Gpdh in *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Mol. Cells.* 11(3), 405-410. 2001.

Phillip, D.P., Childers, W.F., Whitt, G.S. Evolution of patterns of differential gene expression: a comparison of the temporal and spatial patterns of isozyme locus expression in two closely related fish species northern largemouth bass, *Micropterus salmoides salmoides,* and smallmouth bass, *Micropterus dolomieui. J. Exp. Zool.* 210, 473-488. 1979.

Rauchova, H., Zacharova, G., Soukup, T. Influence of chronically altered thyroid status on the activity of liver mitochondrial glycerol 3-phosphate dehydrogenase in female inbred Lewis rats. *Horm. Meta. Res.* 36, 286-290. 2004.

Raymond, J.A. Glycerol and water balance in a near-ososmotic teleost, winter-acclimatized rainbow smelt. *Can. J. Zool.* 71, 1849-1854. 1993.

Raymond, J.A. Glycerol is a colligative antifreeze in some northern fishes. *J. Exp. Zool.* 262, 347-352. 1992.

Raymond, J.A. Glycerol synthesis in the rainbow smelt *Osmerus mordax*. *J. Exp. Biol.* 198, 2569-2573. 1995.

Raymond, J.A. Seasonal variations of trimethylamine oxide and urea in the blood of a cold-adapted marine teleost, the rainbow smelt. *Fish Physiol. Biochem.* 13, 13-22. 1994.

Raymond J.A., Driedzic, W.R. Amino acids are a source of glycerol in cold-acclimatized rainbow smelt. *Comp. Biochem. Physiol.* B. 118, 387-393. 1997.

Raymond, J.A., Hassel, A. Some characteristics of freezing avoidance in two osmerids, rainbow smelt amd capelin. *J. Fish. Biol.* 57, 1-7. 2000.

Raymond, J.A. Hattori, H., Tsumura, K. Metabolic responses of glycerol-producing osmerid fishes to cold temperature. *Fisheries Sci.* 62, 257-260. 1996.

Reshef, L., Olswang, Y., Cassuto, H., Blum, B., Croniger, C.M, Kalhan, S.C., Tilghman, S.M., Hanson, R.W. Glyceroneogenesis and the triglyceride/fatty acid cycle. *J. Biol. Chem.* 278, 30413-30416. 2003.

Richard, J.P. Restoring a Metabolic Pathway. *ACS Chem. Biol.* 3, 605-607. 2008.

Rothe, G.M. *Electrophoresis of enzymes: Laboratory methods*. Springer - Verlag, Germany. 1994.

Salem, M., Kenney, P.B., Rexroad, C.E., Yao, J. Microarray gene expression analysis in atrophying rainbow trout muscle: a unique nonmammalian muscle degradation model. *Physiol. Genomics* 28, 33-45. 2006.

Stroppa, M.M., Carriazo, C., Soria, N., Pereira, R., Gerez de Burgos, N.M. Differential tissue and flight development expression of glycerol 3-phosphate dehydrogenase isozymes in the chaga disease vector *Triatoma infestans*. *Amer. Soc. Trop. Med. Hyg.* 79, 28-35. 2008.

Treberg, J.R. The accumulation, synthetic capacity and intertissue distribution of trimethylamine oxide in deep-sea fish and the cold adapted smelt (*Osmerus mordax*). *Master's Thesis, Memorial University.* 2002.

Treberg, J.R., Lewis, J.M., Driedzic, W.R. Comparison of liver enzymes in osmerid fishes: key differences between a glycerol accumulating species, rainbow smelt (*Osmerus mordax*) and a species that does not accumulate glycerol, capelin (*Mallotus villosus*). *Comp. Biochem. Physiol. A* 132, 433-438. 2002b.

Treberg, J.R., Wilson, C.E., Richards, R.C., Ewart, K.V., Driedzic, W.R. The freeze-avoidance response of smelt *Osmerus mordax*: Initiation and subsequent suppression of glycerol, trimethylamine oxide and urea accumulation. *J. Expe. Biol.* 205, 1419-1427. 2002a.

Tsang, W., Amyes, T.L., Richard, J.P. A substrate in pieces: allosteric activation of glycerol 3-phosphate dehydrogenase (NAD) by phosphite dianion. *Biochem.* 47, 4755-4582. 2008.

Urcelay, E., Jareno, M.A., Menaya, J., Parrilla, R., Ayuso, M.S., Martin-Requero, A. Cloning and functional characterization of the 5' regulatory region of the human mitochondrial glycerol 3-phosphate dehydrogenase gene: lack of 3,5,3'-triiodothyronine responsiveness in adipose tissue. *Eur. J. Biochem.* 267, 7209-7217. 2000.

Walter, J.A., Ewart, K.V., Short, C.E., Burton, I.W., Driedzic, W.R. Accelerated hepatic glycerol synthesis in rainbow smelt (*Osmerus mordax*) is fuelled directly by glucose and alanine: a ^{1}H and ^{13}C nuclear magnetic resonance study. *J. Exp. Zool.* 305A, 480-488. 2006.

Wilanowski, T.M., Yoong, S.H.S., Bartosezewski, S., Gibson, J.B. Expression of the GPDH-4 isozyme of sn-glycerol 3-phosphate dehydrogenase in three *Drosophila* species. *Heredity*, 81, 390-395. 1998.